GENETIC ANALYSIS OF NODULATION AND NITROGEN FIXATION IN THE BROAD-HOST-RANGE *RHIZOBIUM* STRAIN NGR234

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by

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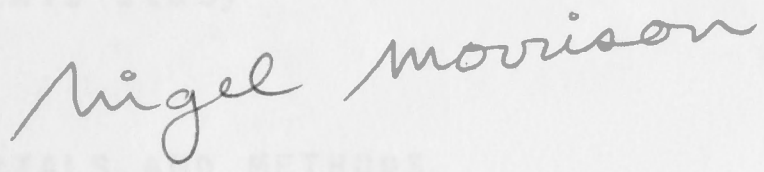
DECLARATION

The research described in this thesis is my own work, except where acknowledgement is made, and has not been submitted for any other degree.



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ABBREVIATIONS

Ap	Ampicillin
Cb	Carbenicillin
Cm	Chloramphenicol
Km	Kanamycin
Rif	Rifampicin
Sm	Spectinomycin
Sp	Streptomycin
Tc	Tetracycline
Nm	Neomycin
Tris	Tris-(hydroxymethyl)aminomethane
kb	kilobase pairs
bp	base pairs
Nod^+	Nodulation-proficient
Nod^-	Non-nodulating
Fix^+	Effective in symbiosis
Fix^-	Ineffective in symbiosis
nif	nitrogenase gene
R	Resistant
S	Sensitive
E.coli	*Escherichia coli*

ABSTRACT

The fast-growing cowpea Rhizobium strain NGR234 is able to nodulate a wide range of tropical legumes as well as the non-legume Parasponia. Nodulation and nitrogenase structural genes are located on a large Sym plasmid in this strain. A cryptic megaplasmid of greater than 450 Mdal also exists in NGR234.

Comparisons between NGR234 and fast-growing R.japonicum strains showed that these organisms are very similar, but NGR234 has a broader host-range. The differences in host-range between these fast-growing strains was similar to the differences between slow-growing cowpea Rhizobium, and R.japonicum strains. The strain NGR234 was only superficially similar to the slow-growing cowpea strain, CP283, which has a near identical host-range to that of NGR234.

The expression of R.leguminosarum and R.trifolii Sym plasmids in strain NGR234 derivatives suggests a closer affinity between NGR234 and classical fast-growing Rhizobium strains which nodulate temperate legumes. Genes which influence the ability of a strain to establish an effective symbiosis, after initial infection were inferred to reside on the non-Sym plasmid complement of NGR234. Similar experiments, using the NGR234 Sym plasmid

transferred into other species of *Rhizobium*, also suggested a role for the genetic background of the *Rhizobium* strain in influencing the success of symbiosis after infection.

The transposon mutagenesis vector, pSUP1011, was used to generate random mutations in NGR234. Plant screening resulted in the isolation of a Nod^- mutant unable to produce root hair curling. This mutant had Tn*5* on the Sym plasmid. Other mutants defective in nodulation had Tn*5* insertions which did not map on the Sym plasmid. These mutants were Hac^+Nod^- or blocked at an early nodulation stage.

pSUP1011 cointegrated randomly into the NGR234 genome at a rate comparable to true Tn*5* transposition. This phenomenon was exploited to isolate a cointegrate between pSUP1011 and the NGR234 Sym plasmid. Since pSUP1011 carries a mobilisation (Mob) site this cointegrate molecule, designated pNM4AN, can be mobilised *in trans* by the plasmid pJB3JI. pNM4AN could transfer to classical fast-growing *R.trifolii*, *R.leguminosarum*, *R.meliloti* and *Agrobacterium tumefaciens* strains at a rate of about 10^{-2}. *R.meliloti* strain ZB157 is a Hac mutant which is Nod^- on lucerne or siratro. Strain ZB157 (pNM4AN) was able to nodulate siratro, producing Nod^+Fix^+ nodules. Microscopy

demonstrated a normal nodule morphology. R.leguminosarum strain 6015 is a deletion mutant, which has lost the ability to nodulate peas. Strain 6015 (pNM4AN) was $Nod^{+}Fix^{-}$ on siratro but was still unable to nodulate peas. R.trifolii strain ANU1064 is a heat-cured Nod^{-} strain which cannot nodulate clovers. Strain ANU1064 (pNM4AN) was $Nod^{+}Fix^{-}$ on siratro but still could not nodulate clover. A.tumefaciens strain C58 carrying pNM4AN was able to form distorted root nodules on siratro. These structures were devoid of bacteria. pNM4AN was stably maintained in all strains. These results show that both nodulation and nitrogen fixation functions for symbiosis with cowpea type plants can be expressed in classic fast-growing Rhizobium strains. Since these transconjugant strains could not nodulate their normal hosts there must be genes on the Sym plasmid which control the host specificity of this broad-host-range cowpea Rhizobium. All attempts to introduce this plasmid into slow-growing cowpea Rhizobium strains were unsuccessful.

Wild-type DNA sequences corresponding to the Sym plasmid Hac mutant were cloned from a lambda Charon-28 gene library of NGR234. It was verified that the correct gene region had been cloned by introducing the cloned fragment, on a mobilisable vector, to the Hac_ mutant. The mutant phenotype was corrected immediately, leading to

a $Nod^{+}Fix^{+}$ response on siratro. A larger fragment (16kb XhoI) did not have all the Sym plasmid encoded Hac or Nod genes from NGR234. This suggests that the organisation of early nodulation genes in NGR234 is different from that seen in R.trifolii where all Sym plasmid encoded Hac and Nod genes map on a 14kb fragment.

CHAPTER ONE

INTRODUCTION

1.1 CLASSIFICATION OF THE *RHIZOBIUM*-LEGUME SYMBIOSIS

The bacterial genus *Rhizobium* is a taxonomic grouping based on the ability of the bacterium to enter a nitrogen-fixing symbiosis with plants of the family Leguminosae. This family of plants is the third largest known, with three subfamilies, numerous tribes and about 16,000 to 19,000 species (Allen and Allen, 1981). Not all species in this family are capable of forming the nitrogen-fixing symbiosis with bacteria and only about 20% of the species have been tested. Most legumes known to be symbiotically proficient fall into the two subfamilies Papilionoidea and Mimosoidea. The first subfamily is the pea-like group and has about 13,000 species and the second is the mimosa-like group with about 2,500 species (Allen and Allen, 1981). It is obvious that within these two subfamilies of a total of about 15,500 species there is room for enormous variation in every conceivable characteristic. The plants themselves include the towering forest giants of the tropics, the characteristic thorn trees of the African veldt and the tiny Arctic *Astragalus* species which still fix nitrogen centimeters

above the permanent frost of the polar regions (Allen and Allen, 1981).

It is not surprising that from this large family of plants a huge array of different types of Rhizobium bacteria have been recorded. These bacteria vary in numerous ways, be it metabolic, physiological, cultural or immunological. There are only two factors which unify all these diverse types of bacteria into one genus; they are all Gram-negative and they all form nodules on the roots of a member of the Leguminosae. From the complexity of this chaotic situation, research through this century has lead to an ordered system of classification. This system is based on the "cross-inoculation group" concept.

This concept is based on practical experience derived from thousands of plant infection tests by many workers. Experience dictates that rhizobia isolated from a particular plant will nodulate that plant again, as well as a range of other plants defined as belonging to a cross-inoculation group. This concept has been applied to many easily accessible legumes over the years and has lead to the classification of the Rhizobium genus into species. For example, a strain of bacteria isolated from a clover (Trifolium) species will nodulate a number of clover species and is therefore regarded as R.trifolii. The major cross-inoculation groups and species of Rhizobium are shown in Table 1.1.

There are many examples of certain strains of

bacteria which can cross the boundaries of this classification system to give an effective nitrogen-fixing symbiosis on a plant of another cross-inoculation group, and this has lead some authors (Wilson, 1944) to question the validity of this system. However, as a general rule it holds, since the majority of strains within a cross-inoculation group will interact only poorly with plants of another group.

As more research is done on the vast number of legumes present throughout the world there are certain to be new groups and a reordering of others, especially the cowpea miscellany. Already Trinick (1968) has revealed a new group of rhizobia which nodulate the tropical coffee shade tree Leucaena leucocephala.

1.2 NODULE MORPHOGENESIS[1]

The development from root hair to functional nitrogen fixing nodule is an exceedingly complex organogenic pathway which is not fully understood biochemically and genetically. Comprehensive reviews have been published (Dart, 1977; Vincent, 1980; Bauer, 1981) which describe the manner in which symbiosis develops between the bacterium and the plant. A phenotypic key devised by Vincent (1980) to classify the stages of infection which are apparent from cytological and cross-inoculation studies will be used throughout this thesis (see Fig.

[1] Referring to plants which have the infection thread invasion mechanism, rather than the lateral root primordia invasion mechanism described by Chandler (1978) and Chandler et al., (1982).

1.1).

The first stage of infection is the attachment of bacterial cells to the root hair. Considerable controversy exists about whether this interaction is host specific and is mediated by plant lectins (Dazzo and Hubble, 1975a; Dazzo and Hubble, 1975b; reviewed by Dazzo and Truchet, 1983). At least in R.trifolii, a polar binding of the bacterium to the side of the root hair occurs soon after colonisation, but again it is not known if this significant. Bhuvaneswari et al. (1980) found with soybean that early root epidermal cells, which had not yet developed root hairs, were the most susceptible to infection and subsequent nodulation. This also held true for the cowpea (Bhuvaneswari et al., 1981) and siratro, (R. Ridge, personal communication) the test plant used in this thesis. Concurrent with colonisation the phenomenon of root hair curling occurs. This is characterised by an extreme curling of the tip of the root hair back on itself to form a "shepherd's crook". Other morphological distortions of root hairs can occur, such as bifurcation, branching and swelling of the ends. The "shepherd's crook" type of curling is thought to be indicative of infection, and again it is unknown whether this phenomenon is host specific, since heterologous Rhizobium species have been observed to cause varying degrees of root hair curling on plants of other cross inoculation groups (Dart, 1977; Yao and Vincent, 1969). The function of root hair

curling seems to be to trap a Rhizobium cell or microcolony in a small pocket between two opposing root hair cell walls. In this microenvironment, the Rhizobium degrades the cell wall of the root hair and penetrates the cytoplasm (Callaham and Torrey, 1981). The plant responds by elaborating the infection thread, a hypha -like growth of normal cell wall components, which grows down the root hair and penetrates the cortex. The Rhizobium cells apparently divide when inside the infection thread, giving the impetus for further growth of the threads towards the cortex. The root hair cell nucleus guides the thread along a dominant cytoplasmic strand in the root hair, and the thread eventually reaches the base of the root hair cell. It is not clear just how the nucleus, which is ahead of the infection thread, manouvers the thread to fuse with the cell wall at the base of the infection thread. It is also unknown how the thread passes from one plant cell to another, although it seems likely that bacteria degrade both cell walls and re-initiate the infection thread. Rhizobium cells have been observed to form microcolonies between the cell walls at the site of re-initiation of the infection thread. Presumably such microcolonies mimic the situation which occurs in the small pocket where the rhizobia are trapped by the curled root hair.

Ahead of the infection thread, cortical cells acquire meristematic activity, swelling and dividing to give the

first indication of a nodule. In round nodules (like soybean and cowpea) the meristematic activity occurs throughout the developing nodule. Bacteria are released from the infection threads in the centre of the nodule, and these infected cells are thought to continue dividing until nodule maturity to form a central core of nitrogen fixing cells. The meristematic activity then ceases, giving the so called determinate nodule. Clovers, peas and many other plants have indeterminate nodules, where the meristematic activity forms a hemispherical focus which keeps growing at the tip of the nodule giving cylindrical nodules. Bacteria are released from infection threads into central cells, below the meristematic cap zone. Infected cells develop to metabolically support the nitrogen-fixing bacteroids, but they do not divide. The determinate and indeterminate nodules also have differences related to vascular supply and metabolism of nitrogen into a transport compound. Determinate nodules usually produce ureides (allantoin, allantoic acid) apparently from specialised uninfected cells within the nodules (Hanks et al.,1983). Indeterminate nodules produce amides (glutamine, asparagine) as nitrogen transport compounds.

Release of bacteria from infection threads occurs from a vesicle-like structure which forms at the end of an infection thread after penetration of a cell. This vesicle seems to be comprised of the zoogloeal matrix of

the thread interior, surrounded by the plant cell membrane. The mechanism by which synthesis of the infection thread is replaced by the development of the release vesicle is not known.

Bacteria are budded off the release vesicle into the host cytoplasm, and are surrounded by membranes derived from the plasmalemma. Subsequently, the bacteria differentiate into pleomorphic nitrogen fixing bacteroids. In determinate nodules the bacteroids are slightly swollen rods, rather than the grossly -pleomorphic forms seen in indeterminate nodules (pea, clover and so on). Certain plant responses are induced at this stage, particularly the production of nodulins (nodule specific proteins) such as the apoprotein leghaemoglobin (Fuller et al., 1983).

It is not known exactly where in the developmental sequence of nodulation that host specific interactions occur. Root hair curling and deformation do not appear to be strictly host specific although there may be a degree of specificity between plants of different sub-families (Yao and Vincent, 1969). Actual root hair curling and deformation may not be strictly necessary for nodulation since many plants which nodulate evince no root hair curling (including Parasponia, described below) and probably have alternative infection strategies like peanut (Dart, 1977). Root hair curling is probably an adjunct to infection, acting to increase the probability of trapping

Rhizobium cells in a site favourable for penetration into the plant. This penetration step may be host specific although little is known about this stage in nodule development. Heterologous rhizobia which are able to curl the root hairs of plants of another cross-inoculation group are reportedly unable to form infection threads (Yao and Vincent, 1969).

Even if a bacterium can invade the root and initiate a nodule there are still host specific interactions at the stage of induction of a nitrogen-fixing symbiosis. Differences in effectivity between bacterial strains and closely related plants is a common phenomenon. Early senescence or active degeneration of plant cell material is associated with an ineffective nodule.

1.3 FAST- AND SLOW-GROWING RHIZOBIA

The diverse bacteria which constitute the genus *Rhizobium* can be divided into so-called fast-growers and slow-growers. These groups have been defined by their rate of growth on laboratory media; fast-growers have a generation time of two to five hours whilst that of slow-growers is greater than ten hours. This distinction may seem arbitary, and there are *Rhizobium* strains which are of intermediate growth, however other physiological and taxonomic differences exist to make classification unambiguous (Jordan,1982). The most often used criterion

is that slow-growers are unable to utilise disaccharide sugars such as sucrose, lactose and maltose. Slow-growing Rhizobium strains are taxonomically similar to Pseudomonas and attempts have recently been made to give them generic status as Bradyrhizobium (Jordan,1982).

The endosymbionts of clovers, peas, Phaseolus beans and medics are characteristic fast-growing rhizobia. Slow-growing organisms have not been reported from these plant hosts. The Lotus group of plants are nodulated frequently by both fast and slow-growing rhizobia (Nutman, 1956), and this is still the only example of an equal representation of both fast- and slow-growers in a cross-inoculation group.

The slow-growing rhizobia dominate the soybean cross-inoculation group and the so-called "cowpea miscellany". The soybean group is defined by the nodulation of a single species (Glycine max), while the "cowpea miscellany" is a remarkable array of cross-infective plants and bacteria from tropical vine, herb, shrub and tree species of all three legume subfamilies (Mimosoideae, Ceasalpinoideae and Papilionoideae). The indicator for this group is a very promiscuously nodulating plant, the cowpea (Vigna unguiculata) (Allen and Allen, 1981). The soybean group is really a subset of the cowpea group since R.japonicum can effectively nodulate some cowpeas and "cowpea" rhizobia can sometimes effectively nodulate soybeans. The taxonomic positioning of soybean in the sub-tribe

Glycininae, tribe Phaseolinae emphasises how it is related to cowpea plants, since all other genera in the Phaseolinae (except the enigmatic Phaseolus) are nodulated by cowpea rhizobia.

Phaseolus is a largely temperate genus which is closely related to Vigna and many other "cowpea" plants, so much so that there are great difficulties of classification. Phaseolus is nodulated exclusively by fast-growing organisms, yet the plant is obviously linked with "cowpea" plants. The R.phaseoli organisms are extremely similar to R.trifolii and R.leguminosarum (Graham, 1964; Hombrecher et al., 1981; Johnston and Beringer, 1977; Beynon et al., 1980). It seems that temperate habitats are dominated by fast-growing organisms and a genus such as Phaseolus, which is presumably a migrant from tropical zones, must adapt to the dominant soil microflora. Perhaps the postulate of Norris (1956) is correct, that legumes evolved in tropical zones, with a broad-host-range endosymbiont and subsequent migration to temperate regions lead to the evolution of precise host specific systems. In the light of this concept it would be interesting to re-examine the symbionts of tropical clover or pea varieties to see if slow-growing R.trifolii or R.leguminosarum exist.

Norris' postulate also suggests that slow-growing rhizobia are ancestral forms and that fast-growing rhizobia have evolved more recently, either by independent

evolution or by acquiring the symbiotic trait from slow-growing rhizobia. The presence of the symbiotic genes on extrachromosomal elements (plasmids, discussed below) in fast-growing rhizobia suggests a facet of evolutionary diversity since plasmids have been implicated in rapid evolutionary change in bacteria. Plasmids have not been demonstrated to carry symbiotic genes in any slow-growing Rhizobium species, so with these organisms the symbiotic trait appears to be chromosomally determined.

1.4 NODULATION OF PARASPONIA

The genus Parasponia belongs in the family Ulmaceae (elm trees). Nodules were first observed on Parasponia by Ham (1909) (described by Akkermans et al., 1978) although this report was lost for many years. Trinick (1973) conclusively proved that the nodules were induced by an endosymbiotic bacterium with the characteristics of Rhizobium. Isolates from Parasponia nodules were able to infect Parasponia plants inducing nitrogen-fixing nodules, thereby fulfilling Koch's postulate. Parasponia plants cultivated aseptically do not develop nodules.

In studies of the position of Parasponia in the cross-inoculation group concept, Trinick and Galbraith (1980) made the startling discovery that some of the Parasponia bacteria could nodulate a number of legumes from the

cowpea group, with varying degrees of effectiveness. Similarly "classic" cowpea Rhizobium strains, such as CB756 (a widely used commercial inoculum strain) could nodulate Parasponia. The endosymbiont of Parasponia was therefore considered a Rhizobium species. Although most Parasponia Rhizobium strains were not highly effective in symbiosis with cowpea plants one strain (CP283) was able to effectively nodulate a wide range of cowpea plants. Trinick (personal communication) believes that this strain represents a chance isolation of a normal cowpea Rhizobium strain from Parasponia.

Parasponia can also be nodulated by slow-growing R.japonicum and R.loti strains as well as numerous fast-growing rhizobia from Leucaena and Mimosa. On the basis of this evidence, Trinick assigned the Parasponia -Rhizobium symbiosis to the cowpea miscellany. This does not mean that all cowpea rhizobia will nodulate Parasponia. The reverse is also true since a number of Parasponia Rhizobium strains have a restricted host-range, seeming to be specialised for Parasponia.

The mode of bacterial infection in Parasponia is not fully understood. It is certain that root hair curling and distortions do not occur. Nodule sites are not predominantly at the lateral root axils as in peanut (Chandler, 1978; P. Dart, personal communication), and Stylosanthes (Chandler et al.,1982) where a mode of infection occurs which does not utilise root hairs. The

Parasponia nodule has an internal structure similar to nodules on another non-legume, Alnus, (Becking, 1977) where the bacteroid zone exists as a sheath around a central vascular system resembling the organisation of a lateral root. Infection threads occur within the nodule but have not been observed in root hairs. In legumes, bacteria are released from infection threads by budding off a release vesicle which forms after penetration of the thread into a cell destined to contain bacteroids. In Parasponia the infection thread wall changes in structure to become a continuous "fixation thread" which develops throughout the cell. NGR234 is capable of reaching this stage of infection (M.J. Trinick, personal communication) but is unable to initiate nitrogen fixation. Although Parasponia nodules contain no leghaemoglobin (Coventry et al., 1976) a haemoprotein of some type (Appelby et al., 1983) is present to provide the oxygen-buffering capacity necessary for nitrogen fixation.

1.5 GENETICS OF RHIZOBIUM

The genetics of Rhizobium has developed rapidly over the last decade. Research has been centered on the fast-growing species R.trifolii, R.leguminosarum and R.meliloti. More recently, slow-growing cowpea and R.japonicum strains have come under study.

Early research used chemical mutagenesis in

comprehensive studies of physiological, biosynthetic and metabolic pathways in R.leguminosarum and R.meliloti (reviewed by Schwinghamer, 1977). Circular linkage maps quickly developed for these two species and R.trifolii as well, when chromosome-mobilising plasmids were discovered in Pseudomonas (Haas and Holloway, 1976; Holloway, 1979). The linkage maps indicated a similarity between R.leguminosarum and R.trifolii which did not extend to R.meliloti (Kondorosi et al., 1980). Surprisingly few symbiotically-defective mutants could be mapped by this method.

Many auxotrophic mutants isolated during this phase of Rhizobium genetics were tested for symbiosis as an indirect way to investigate the requirements for nitrogen fixing symbiosis. The most striking result was the strong correlation between adenine auxotrophy and an inability to fix nitrogen (Pankhurst and Schwinghamer, 1974; Denarie et al., 1976; Pain, 1979). Other auxotrophic requirements were variably associated with symbiotic deficiency. However in these studies it was impossible to know if any single mutagenic events had occurred, since the auxotrophic mutants were isolated from among survivors of the mutagenic treatment. The correlation of the mutant phenotype with the symbiotic deficiency can be verified by the analysis of revertants, if they can be isolated.

The problems associated with the chemical mutagenesis were alleviated by the introduction of transposon

mutagenesis (Beringer et al., 1978a).

Transposons are discrete DNA entities which share with insertion sequences the ability to translocate from one site in a DNA molecule to another, with little specificity (Kleckner, 1977; Starlinger, 1980). The insertion of a transposon at a given site usually leads to the inactivation of whatever gene resides at that locus, although other effects can occur, such as enhanced gene expression, or relief of normal gene control mechanisms due to promotor-like sequences present in the ends of certain transposons (Kleckner, 1981). If the transposon has inserted in an operon comprised of a number of genes a "polar" effect may occur. This means that genes "down-stream" from the transposon with respect to the site of mRNA initiation (the promotor) are inactivated (probably because transcription stop signals occur in the transposon) while the "upstream" genes are still expressed. Polar effects do not always occur (Berg et al., 1980), presumably due to lack of mRNA termination, or the activity of promotor-like sequences in the ends of the transposon.

Early experiments showed that the bacteriophage Mu, a transposable element long used in E.coli genetics, was unsuitable in Rhizobium (Van Vliet et al., 1978). From these experiments developed the concept of a suicide plasmid, since conjugative plasmids containing Mu could

not become established properly in Rhizobium. Beringer et al. (1978a) loaded the transposon Tn5 onto a suicide plasmid and discovered that the Tn5 element could transpose in R.leguminosarum. The suicide plasmid was subsequently lost from the Rhizobium cell. Tn5 was chosen because it has little preference for particular DNA sites in E.coli, and the aminoglycoside antibiotic resistance determinants (Km and Nm resistance) are easily selected in Rhizobium. Since a mutated cell can be selected for by the Km^R phenotype, one can be sure that only mutated clones are chosen for the laborious screening needed to identify symbiotically defective mutants. The Km^R acts as a genetic flag for the locus of a particular mutated gene so linkage mapping is simplified since the Km^R phenotype replaces the individual plant tests required to measure linkage frequencies. Because Tn5 lacks recognition sites for a number of restriction endonucleases (Jorgensen, 1979), it is possible to isolate DNA fragments which contain Tn5 and surrounding Rhizobium DNA out to the first restriction site on either side of the Tn5. These "mutated" fragments can be used to find corresponding unmutated (wild-type) fragments from gene libraries (Scott et al., 1982). This method allows the analysis of genes at the DNA level.

While the transposon mutagenesis system provided the method to identify specific genes involved in general metabolism and symbiosis, the study of Rhizobium plasmids

answered questions concerning the location and arrangement of genes responsible for symbiosis. Higashi (1967) was able to transfer clover infectivity from R.trifolii to R.phaseoli. This ground-breaking work demonstrated that bacterial host-specific nodulation determinants are controlled or encoded on an episomic (plasmid) element. Much later, other workers verified that plasmids are abundant in fast-growing Rhizobium strains and that, in nearly all cases, one of these plasmids carries genes responsible for both nodulation and nitrogen fixation. These plasmids are now commonly called Sym plasmids (Casse et al., 1979; Hooykaas et al., 1981).

A general method of curing, or eradicating, plasmids in R.trifolli by the use of high incubation temperatures (Zurkowski and Lorkiewicz, 1978,1979) demonstrated that plasmid control of nodulation was a general characteristic of R.trifolii. Zurkowski (1980,1981) showed that loss of many characteristics of early infection of clover by R.trifolii was correlated with the loss of a single plasmid. When this plasmid was reintroduced all symbiotic properties were restored. Hooykaas et al. (1981) transferred a plasmid from R.trifolii to Agrobacterium tumefaciens and showed that early nodulation functions were expressed. Hirsch et al. (1980) correlated loss of nodulation ability in R.leguminosarum with deletions in certain plasmids. Brewin et al. (1980a, 1980b) demonstrated that genes for

host-specific nodulation and nitrogen fixation in certain R.leguminosarum strains were carried on transmissible plasmids which also encoded bacteriocin production. Hombrecher et al. (1981) showed that both nitrogen fixation genes and nodulation genes were linked in R.leguminosarum and R.phaseoli . In R.meliloti, Casse et al. (1979) demonstrated very large plasmids which later were shown to carry genes essential to nitrogen fixation and nodulation (Rosenberg et al., 1981; Banfalvi et al., 1981). Studies on the location of genes for nitrogenase were facilitated by the discovery of Ruvkun and Ausubel (1980) that Klebsiella pneumoniae nifH and nifD genes can be used to identify homologous genes in Rhizobium.

Mapping of Rhizobium plasmids on the DNA level (Prakash et al., 1981; Kondorosi et al., 1982; Schofield et al., 1983a; Pankhurst et al., 1983) has led to the general conclusion that genes involved in symbiosis are clustered on a large segment of Sym plasmids. Nodulation genes map about 20kb from structural nitrogenase genes (nifH and nifD), and other genes involved in nitrogen fixation and nodule development also map in this symbiotic region (Ma et al., 1982; Downie et al., 1983b). The analysis of the nitrogenase operon is well advanced (Ow and Ausubel, 1983; Zimmerman et al., 1983; Corbin et al., 1983; Sundaresan et al., 1983) with the discovery that a Klebsiella regulatory gene (nifA) can activate Rhizobium nifH promotors. Analysis of nodulation genes has lagged

behind the *nif* gene story, probably because the *Klebsiella nif* model has provided many answers for *Rhizobium nif* studies but no conceptually similar system as nodulation has been studied before.

1.6 THE ORGANISM, NGR234

The first fast-growing *Rhizobium* strain to be recognised as a cowpea *Rhizobium* was NGR234, isolated by Trinick in New Guinea from the fodder legume *Lablab purpureus*. This strain was able to effectively nodulate a large number of the "cowpea" legumes and was also able to fix nitrogen with *Leucaena leucocephala* (a member of the Mimosaceae). In addition, NGR234 could form nodules on soybean (*Glycine max*) and *Parasponia*. The known host-range of NGR234 is shown in Table 1.2. This host-range extends over two sub-families of the Leguminosae and many sub-tribes. NGR234 was the only fast-growing strain isolated from 30 *Lablab purpureus* nodules collected at the same site, and was the only fast-grower from over 250 isolations made by Trinick (1980) from plants normally nodulated by slow-growing rhizobia. Trinick (1980) describes NGR234 as a typical fast-grower in cultural habit and growth in litmus milk. NGR234 had a high immunological cross reactivity with a *L.leucocephala Rhizobium* strain NGR99, but did not react with four other *Leucaena* strains.

Other fast-growing cowpea strains have recently been identified from India (P. Dart, personal communication) and fast-growing R.japonicum strains have emerged from China (Keyser et al., 1981). The significance of these strains in the evolution of Rhizobium is tantalising and certainly worthy of study.

1.7 AIMS OF THIS STUDY

The genetic analysis of symbiosis was already well advanced in R.trifolii, R.leguminosarum and R.meliloti when this study began. Nothing was known concerning the genetics of symbionts of the cowpea miscellany and Parasponia.

Differences between the fast- and slow-growers were well known in a cultural sense but had not been investigated to any degree in the genetic sense. The strain NGR234 seemed to encompass all these problems, being a fast-growing cowpea Rhizobium with an unusually broad-host-range, which crosses several cross-inoculation boundaries, extending to the non-legume Parasponia. The aims of this study were therefore:

1. To make the strain NGR234 amenable to genetic study and to bring it to the stage reached with R.trifolii, R.leguminosarum and R.meliloti.
2. To investigate the control of nodulation by introducing plasmids from other Rhizobium species or

transferring the nodulation ability of NGR234 to other rhizobia.

3. To isolate mutants blocked at various stages of symbiosis, and to compare the nodulation of tropical legumes with temperate legumes.

4. To isolate genes essential for the broad-host-range nodulation phenotype, ultimately to determine whether the same genes are required for different infection strategies on legumes and Parasponia.

Table 1.1 Cross inoculation groups used to classify the *Rhizobium*-legume symbiosis.

RHIZOBIUM SPECIES	GROWTH RATE	HOST AFFINITIES	PLANT GROUP
R.meliloti	fast	*Melilotus*, *Medicago* *Trigonella*	Alfalfa
R.trifolii	fast	*Trifolium*	Clover
R.leguminosarum	fast	*Pisum*, *Lathyrus*, *Lens*, *Vicia*	Pea
R.phaseoli	fast	*Phaseolus*	Bean
R.loti	fast and slow	*Lotus*	Trefoil
R.lupini	slow	*Lupinus*, *Ornithopus*	Lupin
R.japonicum	slow	*Glycine*	Soybean
Rhizobium species	slow	many genera	Tropical beans

Table 1.2 Results of known host-range tests on strain NGR234.

TRIBE	SUB-TRIBE	GENUS SPECIES	RESPONSE[1] Nod	Fix	SOURCE[2]
1. Sub-family: Papilionoideae					
Phaseoleae	Phaseolinae	Lablab purpureus	+	+	1
		Macroptilium atropurpureum	+	+	1, 2
		Macroptilium lathyroides	+	+	1, 2
		Psophocarpus tetragonolobus	+	+	3
		Vigna unguiculata	+	+	1, 2
		Macrotyloma axillaris	-		2
		Phaseolus vulgaris	-		1, 2, 3
	Diocleinae	Calpogonium caeruleum	+	+	1
	Clitoriinae	Centrosema pubescens	+	+	1
	Cajaninae	Flemingia congesta	+	+	1
	Glycininae	Glycine wightii	+	+	1, 2
		Glycine ussuriensis	+	+	1, 2
		Glycine tabacina	+	-	2
		Glycine tomemtella	-		2
		Glycine max cv. Bragg	+	-	2
		Glycine max	+	+	1
		Teramnus uncinatus	+	e	2

Table 1.2 Continued.

Desmodieae	Desmodium intortum	+	+	2, 3
	Desmodium uncinatum	+	e	2
Tephrosieae	Tephrosia candida	+	+	1
Aeschenomenae	Stylosanthes hamata	+	+	3
	Stylosanthes humilis	-		2
	Arachis hypogea	+	-	2, 3
Robineae	Sesbania grandiflora	-		1
Bossiaea	Crotolaria pubescens	-		1
Genisteae	Lupinus augustifolins	-		1
Vicieae	Pisum sativa	-		1, 2
Trifolieae	Trifolium repens	-		1, 2
	Trifolium subterraneum	-		2
	Trifolium dubium	-		2
	Medicago sativa	+		1
		-		2
2. Sub-family Mimosoideae				
Mimoseae	Mimosa invisa	-		1
	Leucaena leucocephala	+	+	1, 2
Acacieae	Acacia farnesiana	+	-	1

Table 1.2 Continued.

3. Sub-family Caesalpinioideae				
Cassieae	Cassia tora	-		1
4. Non-legumes				
Family Ulmaceae	Parasponia andersonii	+	-	2, 4

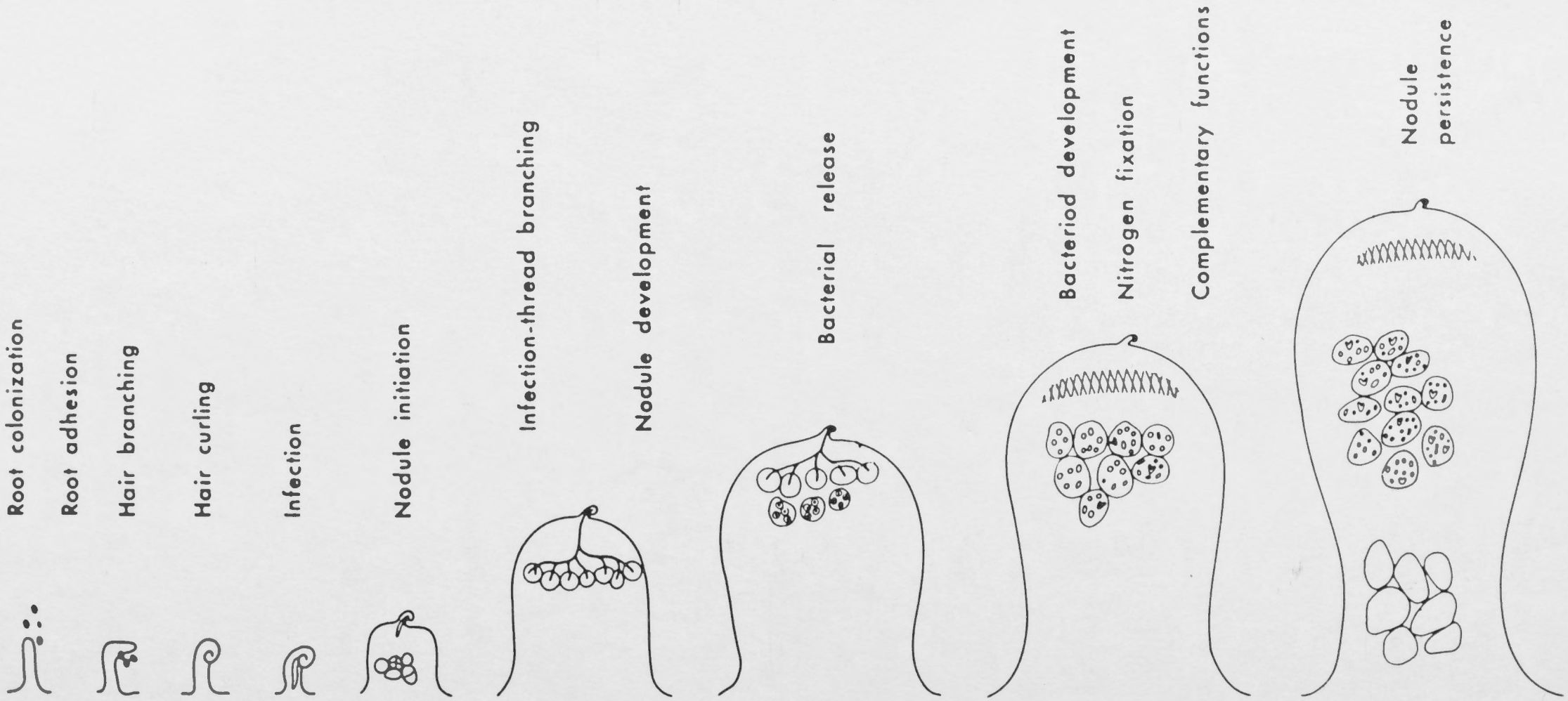
NOTE

1. Nod^+ means nodulation, Nod^- means no nodulation. Fix refers to nitrogen fixation; + is fully effective, e is partially effective, - means no nitrogen fixation.
2. Sources : 1. Trinick (1980) ; 2. Personal observations; 3. W. Broughton, personal communication; 4. Trinick and Galbraith (1980)

Taxonomic classification follows that described by Allen and Allen (1981), and Goldblatt (1981).

Figure 1.1. A phenotypic key devised by Vincent (1980) and Rolfe *et al.* (1981a) to describe the stages of the infection process.

Roc	Roa Hab Hac	Hac	Inf	Inf Noi	Inb	Nod	Nod Bar	Bad Nif Cof	Nif Cof Nop



CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Bacterial strains, plasmids and vectors

Bacterial strains are listed in Table 2.1. Plasmids and vectors used are listed in Table 2.2. Plasmids constructed during this thesis are listed in Table 2.3.

2.1.2 Plant species and varieties

These are listed in Table 2.4.

2.1.3 Media

Luria Broth (LB) (Miller, 1972)

Bacto-tryptone	10 g l^{-1}
Yeast extract	5 g l^{-1}
NaCl	5 g l^{-1}
pH	7.5

LM

As for LB but with the addition of

maltose	2 g l^{-1}
1M $MgCl_2$	10 ml l^{-1}
1M Tris pH 7.4	10 ml l^{-1}

Gamborg's Trace Elements Solution

(Gamborg and Eveleigh, 1968)

$MnSO_4 \cdot 4H_2O$	10 g l^{-1}
H_3BO_3	3 g l^{-1}
$ZnSO_4 \cdot 7H_2O$	3 g l^{-1}
$Na_2MoO_4 \cdot 1H_2O$	250 mg l^{-1}
$CuSO_4 \cdot 5H_2O$	250 mg l^{-1}
$CoCl_2 \cdot 6H_2O$	250 mg l^{-1}

Gibson's Trace Elements Solution (Gibson, 1963)

H_3BO_3	2.86 g l^{-1}
$MnSO_4 \cdot 4H_2O$	2.03 g l^{-1}
$ZnSO_4 \cdot 7H_2O$	220 mg l^{-1}
$CuSO_4 \cdot 5H_2O$	80 mg l^{-1}
$H_2MoO_4 \cdot 1H_2O$	90 mg l^{-1}

Fahreaus Medium (F) (modified) (Vincent, 1970)

$CaCl_2 \cdot 2H_2O$	100 mg l^{-1}
$MgSO_4 \cdot 7H_2O$	120 mg l^{-1}
KH_2PO_4	100 mg l^{-1}
$Na_2HPO_4 \cdot 12H_2O$	150 mg l^{-1}
Ferric citrate	1.5 mg l^{-1}
Gibson's trace elements	1 ml l^{-1}
pH	6.5

Bergersen's Modified Medium (BMM) (Bergersen, 1961)

$Na_2HPO_4 \cdot 12H_2O$	360 mg l^{-1}
$MgSO_4 \cdot 7H_2O$	80 mg l^{-1}
$FeCl_3$	3 mg l^{-1}
$CaCl_2$	40 mg l^{-1}
Mannitol	3 g l^{-1}
Thiamine	2 mg l^{-1}
Biotin	0.2 mg l^{-1}
Sodium glutamate	500 mg l^{-1}
Yeast extract	500 mg l^{-1}
Gamborg's trace elements	1 ml l^{-1}
pH	7.0

BMM plus Mannitol (BMM + man)

(Gresshoff and Rolfe, 1978)

As for BMM but with the addition of 36 g l^{-1} mannitol.

Tryptone Yeast (TY) (Beringer, 1974)

Bacto-tryptone	5 g l^{-1}
Yeast extract	3 g l^{-1}
$CaCl_2 \cdot 2H_2O$	0.9 g l^{-1}
pH	7.0

Trifolii Medium Yeast (TMY)

(Skotnicki and Rolfe, 1979)

$(NH_4)_2SO_4$	250 mg l^{-1}
KH_2PO_4	150 mg l^{-1}
$MgSO_4$	200 mg l^{-1}
NaCl	100 mg l^{-1}
$NaMoO_4$	25 mg l^{-1}
$CaCl_2 . 2H_2O$	250 mg l^{-1}
$FeSO_4 . 7H_2O$	2.5 mg l^{-1}
Sucrose	5 g l^{-1}
Arabinose	5 g l^{-1}
Thiamine	1 mg l^{-1}
Biotin	0.5 mg l^{-1}
Nicotinic acid	1 mg l^{-1}
Pyridoxine-HCl	1 mg l^{-1}
Gamborg's trace elements	1 ml l^{-1}
Yeast extract	1 g l^{-1}
pH	7.1

Trifolii Minimal Medium (TM)

As for TMY but without yeast extract.

Trifolii Minimal Medium Sucrose (TMS)

As for TM but without arabinose.

Soft agar

1 M $MgSO_4$	10 ml l^{-1}
agar	10 g l^{-1}

All autoclaving was done at 15psi for twenty minutes at 120°C.

2.1.4 Antibiotics

For Rhizobium strains the following concentration of antibiotics were used:

Carbenicillin (Cb)	75 ug ml^{-1}
Chloramphenicol (Cm)	15 ug ml^{-1}
Kanamycin (Km)	200 ug ml^{-1}
Streptomycin (Sm)	200 ug ml^{-1}
Spectinomycin (Sp)	200 ug ml^{-1}
Rifampicin (Rif)	50 ug ml^{-1}
Tetracycline (Tc)	4 ug ml^{-1}

For E.coli strains the following concentrations of antibiotics were used:

Cm	15 ug ml^{-1}
Km	50 ug ml^{-1}
Rif	50 ug ml^{-1}
Sm	250 ug ml^{-1}
Mercuric Chloride	12 ug ml^{-1}

2.1.5 Buffers

a. Electrophoresis buffers: Tris-acetate-EDTA (TAE) buffer was routinely used for agarose gel electrophoresis of DNA. The buffer was prepared as a concentrate and diluted twenty times prior to use.

TAE

Tris-acetate	40 mM
Sodium acetate	5 mM
Ethylenediamine tetraacetic acid (EDTA)	1 mM
pH	7.8

Gel sample buffer

Glycerol	80 mg ml^{-1}
EDTA	100 mM
Bromophenol Blue	0.3 mg ml^{-1}
pH	8.0

b. Buffers for enzymology: TA buffer (O'Farrell et al., 1980) was used for all restriction endonuclease reactions. A ten times concentrate was prepared by mixing 0.8 ml of solution A and 0.1 ml each of solution B and C, where;

Solution A

Tris-acetate	5.0 mg ml^{-1}
Potassium acetate	8.1 mg ml^{-1}
Magnesium acetate	2.5 mg ml^{-1}
pH	7.9

Solution B

Dithiothreitol (DTT)	50 mM

Solution C

Bovine serum albumin (BSA)	10 mg ml^{-1}

The final concentrations used in restriction reactions were;

Tris-acetate	33 mM
Potassium acetate	66 mM
Magnesium acetate	10 mM
DTT	500 uM
BSA	100 ug ml^{-1}

HaeIII buffer was used for ligation reactions and making radioactive probes. A ten times concentrate was prepared as follows;

Tris-HCl	60 mM
Magnesium chloride	100 mM
Mercaptoethanol	100 mM
pH	7.6

TE buffer was used for storing DNA;

Tris-HCl	10 mM
EDTA	1 mM
pH	8.0

TES buffer was TE with 100 mM sodium chloride.

c. Buffers for Southern hybridisation:

DNA denaturation buffer

Sodium hydroxide	0.5 M
Sodium chloride	0.5 M

Tris-salt buffer

Tris-HCl	0.5 M
Sodium chloride	2.0 M
pH	7.2

Standard saline citrate (SSC), 20 x concentrate

Sodium chloride	3.0 M
Tri-sodium citrate	0.3 M

Hybridisation buffer

Sodium dodecylsulphate (SDS)	0.1 mg ml^{-1}
Herring sperm DNA (Sigma)	0.2 mg ml^{-1}
Polyvinylpyrrolidene	0.2 mg ml^{-1}
Hepes buffer (Sigma)	50 mM

Ficoll-400 (Sigma)	0.2 mg ml^{-1}
20 x SSC	150 ml l^{-1}
BSA	0.2 mg ml^{-1}
pH	7.0

2.1.6 Enzymes

Restriction endonucleases were purchased from New England Biolabs, Inc., Bethesda Research Laboratories Inc. or Boehringer Mannheim and used according to the manufacturer's directions. DNA polymerase I (both the Klenow fragment and the holoenzyme) was obtained from New England Biolabs or Boehringer Mannheim. Lysozyme, Ribonuclease A (RNA'ase) and Deoxyribonuclease I (DNA'ase) were obtained from the Sigma Chemical Co.. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim.

2.2 METHODS

2.2.1 Plant culture

Depending upon the particular plant the method of cultivation varied. The methods used were as follows

a. Plate Culture: This method was used for small legumes such as clovers, Desmodium intortum and siratro. Seeds (excepting clovers) were treated in concentrated sulphuric acid for 5 minutes to scarify the seed coat. They were washed in tap water and then surface sterilised

by treatment for 15 minutes in 10% commercial bleach (Ajax Chemicals, Ltd.). Seeds were washed about ten times in distilled water and placed on plates containing F agar before being covered with soft agar. After the soft agar had set, the plates were wrapped in aluminium foil and stood on their edges slightly off the vertical in a 30°C incubator. The reason for this positioning was to let the seedling root grow away from the agar. The roots are more uniform in size and shape when treated in this manner. It is possible to store ungerminated seeds for up to two weeks at 4°C with no adverse effects. Germinated seeds deteriorate rapidly at 4°C.

Plates containing F agar (30 ml per plate) were prepared for inoculation by first scoring two small parallel furrows about 1 cm apart in the top half of the plate. The seedling roots were placed in these furrows to allow the root to grow down into the inoculum which was applied directly below this point. Plates were sealed with Nescofilm (Bando Chemicals Ltd., Kobe) and a small hole punctured in the film to allow gas exchange. Plates were incubated upright in a growth cabinet with a light intensity of 300 uE m^{-2} min^{-1} at a temperature of 22-24°C with an 18 hour day. Under these conditions nodules developed after two weeks and a nitrogen fixing response (Fix^+) was apparent after eight weeks.

b. Leonard jars: Jars were filled with moistened

sand and pasteurised by heating at 70°C for 2 days, or autoclaved. Assembled jars were covered with reflective paper, and arranged on movable tables in a controlled temperature greenhouse operated at a constant 25°C. Leonard jars were watered with a sterile mineral nutrient solution (F media). All Leonard jar tests were done using five replicates per test. Tables carrying the jars were moved daily within the greenhouse to avoid positional effects. Seeds were sterilised by treatment in 10% mercuric chloride for one minute followed by extensive rinsing. Seeds were then germinated on water agar and planted out when radicles appeared. The inoculum used for these tests was 10 mls of a washed suspension of freshly grown bacteria (about 10^9 cells ml^{-1}).

c. Test tube assays: 30 ml slants of F agar were made in test tubes of 15 by 2.5 cm dimensions. Seedlings with small roots were placed on the surface of the agar and inoculated with bacteria. The tops of the test tubes were then plugged with cotton wool and the plant was not permitted to escape. Roots were kept dark and cultivation was done in the growth cabinet. Leucaena leucocephala assays were done similarly in 100 ml screw cap plastic jars. After one week the plant tops were allowed to protrude from the jar and the jar was sealed with Nescofilm.

d. Glass jars: Two litre glass jars were half-filled with a sterile sand-pearlite mixture and autoclaved. Seeds were planted and inoculated when the radicle was just protruding from the seed coat. The jar lids were removed when the plants were cultivated in the growth cabinet.

e. Pea test: Pea seeds were washed extensively in distilled water and surface sterilised in 1/10 volume commercial bleach. The seeds were washed in sterile water before being placed on water agar plates to germinate. Germinated seeds were transferred to 250 ml conical flasks containing 200 ml of solid F medium and incubated at room temperature in the dark for 2 days. Plants were inoculated with a suspension of a bacterial colony in 10 ml of sterile water. Plants were cultivated in a growth cabinet at a temperature of 16°C with a 12 hour day, and a night temperature of 10°C. The light intensity was 200 uE m^{-2} sec^{-1}. Under these conditions first nodules appeared after two to three weeks and an effective symbiosis was apparent after five weeks.

2.2.2 Acetylene reduction

Whole plant assays were done on small plants. The plant was removed from the culture media and placed in a 28 ml glass scintillation vial with 1 ml of sterile water and a piece of filter paper. The wet filter paper

maintained humidity. The vials were capped with Suba seals which had been autoclaved in detergent, washed and dried overnight at 60°C to drive off any residual ethylene. The gas phase was augmented by the addition of 2 ml of pure acetylene and the vials incubated at 25°C under illumination. Samples of 200 ul were taken at hourly intervals and assayed for ethylene by gas chromatography, using a Hewlett-Packard 5720A gas chromatograph, with a Poropak R-80-100 column at 45°C with nitrogen gas as the mobile phase and a hydrogen flame ionization detection system. Quantitation of ethylene production was done by reference to calibrated standards. Rates were expressed as n mole acetylene reduced per hour per gram fresh nodule weight. Plant top fresh weight could also be used as a measure of symbiotic effectiveness.

Large plants had their root systems excised and placed in 200 ml airtight jars with Suba seals. Acetylene (20 ml) was injected and the production of ethylene monitored as above.

2.2.3 Bacterial matings

a. Filter matings: Recipient and donor strains were grown to mid-log phase on appropriate media either solid or liquid and resuspended in 4 ml of water. Rhizobium recipients were always grown on TY medium, whilst E.coli strains were grown on either TY or LB. Bacterial

suspensions (2 ml) were mixed in a test tube by vortexing and filtered through a sterile 0.45 um nitrocellulose filter (Millipore Inc., Detroit, U.S.A.). The bacteria remained on the filter which was placed on solid medium. For Rhizobium the mating was done on TY medium for 4 hours at 30°C whilst for E.coli it was done at 37°C. The filter was removed from the agar surface after this time and the cells washed off by vortexing in 4 ml of water in a sterile screw-capped bottle. The cell suspension was diluted serially ten-fold and 0.1 ml aliquots plated onto appropriate media to select for transconjugants or to ascertain the viable count of recipient and donor cells. Transfer frequencies were presented as the ratio of transconjugants to total recipient cells. Donor cells were usually at about the same number as recipient cells. This method was used to quantitatively assess plasmid transfer frequencies.

b. Patch matings: This semi-quantitative method was used when transfer frequencies were expected to be high. Donor and recipient cells, grown as described above, were resuspended in 0.5 ml of water. E.coli donors were normally grown on TY medium for crosses with Rhizobium to avoid potential toxicity of the salt in LB medium towards certain rhizobia, notably strain NGR234 derivatives. Donor and recipient cell suspensions (0.05 ml) were mixed in a test tube by vortexing and the mixture dried onto one

half of the surface of a TY plate. Control spots of pure donor and recipient suspensions were dried on the other half of the TY plate. The cultures were incubated overnight at 30°C, before being replica plated onto a series of different media to select for transconjugants, donor alone and recipient alone. The rate of transfer could be estimated through experience by the number of transconjugants which grew. High transfer frequencies gave confluent growth on selective media. Transconjugants from this type of mating had to be rigourously purified on selective media since the chances of donor contamination are higher.

c. Spot matings: This type of mating was used to test for the retransfer of plasmids from a large number of donors (usually sibling transconjugants) to a single recipient. Donor clones were spotted out on appropriate media at a density of 50 per plate and incubated until visible growth occurred. These plates were then replica plated on to lawns of recipient cells. For E.coli recipient the lawns were grown on TY media for 8 hours at 30°C, for Rhizobium lawns were grown for 16 hours. The "mating plates" were incubated at 30°C overnight before being replica plated onto selective media. E.coli transconjugants were selected at 37°C. Control spots of plasmids of known transmissibility were always included. Strains containing transferrable plasmids could easily be

identified since recipient transconjugants only grew on the selective media at positions corresponding to those strains on the master plate.

d. Triparental matings: Some plasmids are not self transferrable (Tra^-) but can be mobilised (Mob+) *in trans* by other plasmids. To effect the transfer of such Tra^-Mob^+ plasmids to *Rhizobium* the triparental mating system of Ditta *et al*. (1981) was used. This system uses two donor strains. The first strain contains the Tra^-Mob^+ plasmid and the other strain contains a Tra^+ plasmid. In these experiments the mobilising plasmid was either pRK2013, which can transfer between *E.coli* strains but cannot replicate in *Rhizobium*, or pED709 (a Km^S RP4 derivative) which can replicate in *Rhizobium*.

These matings were always done by the patch method with the extra combinations of controls to account for the two donor strains. Recipient and donor suspensions were first spotted separately onto one side of a TY plate. Then suspensions of each donor strain mixed separately with the recipient, spotted on a different plate. A combination of the two donors was also done. Lastly a mixture of all three strains was spotted on another plate. The plates at this stage have seven separate combinations of bacteria.

After overnight incubation at 30^oC, plates were replica plated onto appropriate selective media to select for transconjugants.

2.2.4 Isolation of bacteria from nodules

The method of Gresshoff et al. (1977) was used. The nodule was excised from the plant and washed in sterile water several times before being surface sterilised in 1% commercial bleach for 10 to 15 minutes. Nodules were then rinsed free of bleach and transferred to 0.1 ml of protoplast dilution buffer (PDB). Nodules were crushed with a sterile glass rod and the suspension plated out on BMM + man solid medium.

PDB consisted of;

Sorbitol	250 mM
Mannitol	250 mM
Di-potassium hydrogen orthophosphate	2 mM
Calcium chloride	2 mM
pH	5.8

2.2.5 Phenol-chloroform extraction of DNA

Organic solvent extraction was used to remove protein from DNA. The crude DNA preparation was mixed gently by inversion with an equal volume of "phenol" (phenol saturated with TE buffer) and the phases separated by centrifugation. The aqueous phase was extracted again, eventually resulting in a perfectly clear aqueous phase after repeated extractions. The DNA preparation was then extracted with "phenol-chloroform" (an equal volume of

phenol and chloroform) to remove remaining protein. The DNA was then extracted with pure chloroform to remove residual phenol. DNA was then ethanol precipitated by adding 0.1 volume of 3 M sodium acetate pH 6 and 2.5 volumes of ethanol. After mixing, large molecular weight DNA would precipitate at room temperature. Dilute preparations of DNA or small molecular weight DNA had to be frozen at -20°C for one hour or -80°C for 15 minutes prior to recovering DNA by centrifugation. The DNA pellet was washed in 70% ethanol to remove excess salts, then dehydrated in absolute ethanol. Ethanol was removed *in vacuo*. Dry DNA was redissolved in TE buffer to a concentration of 1 mg ml^{-1}, assessed by measuring ultraviolet light absorbance at 260 nanometres. The DNA was stored at 4°C.

2.2.6 DNA isolation

a. Genomic DNA: *Rhizobium* strains were grown in 10 ml of TY medium at 30°C with shaking until mid-log phase, about 30 hours. Cells were pelleted by centrifugation and resuspended in 3 ml of 25% sucrose in TE and protoplasted by the addition of 100 ul of 20 mg ml^{-1} lysozyme in water. After 5 minutes at room temperature the cells were lysed by the addition of 3ml of 0.1% SDS. Proteinase K, 10 mg, was added and the lysate incubated at 65°C for 30 minutes. The lysate was phenol extracted four times with centrifugation to separate the aqueous and organic phases.

The lysate was then extracted four times with phenol-chloroform, followed by four extractions with chloroform alone. DNA was ethanol precipitated, dried *in vacuo* and reconstituted in 1 ml TE buffer.

b. Plasmid DNA: *E.coli* strains carrying amplifiable plasmids were grown in 50 ml LB medium at 37°C with shaking. 10 ml of this culture was used to inoculate into 1 litre of LB media and grown to an optical density of 0.4 at 650 nanometres. Spectinomycin (200 mg) was added to amplify the plasmid. If the plasmid was not amplifiable this step was omitted. After overnight incubation at 37°C with shaking, the cells were harvested by centrifugation[1] at 5,000 rpm for 5 minutes at 0-4°C. The cell pellet was resuspended in 10 ml 25% sucrose in TE and transferred to 40 ml polypropylene tubes. Freshly prepared lysozyme (30 mg in 1 ml of water) was added, followed by 5 ml 0.5 M EDTA pH 8. After 5 minutes the cells were lysed by the addition of 15 ml of 1% Triton-X100. The lysate was centrifuged at 20,000 rpm for one hour at 4°C. The supernatant was decanted and made 3% with respect to sodium chloride and 12.5% with respect to polyethylene glycol 6000 (PEG) and maintained on ice for two hours. The DNA was pelleted by centrifugation at 5,000 rpm for 5 minutes. The pellet was dissolved in 5 ml TES buffer and then 8 g of caesium chloride was added. The caesium chloride was dissolved by gentle rocking of the tube and

[1] Unless otherwise specified, a Sorvall SS-20 rotor was used in a Sorvall RC-5B centrifuge.

the solution was centrifuged at 18,000 rpm for 30 minutes. A pellicle of PEG was removed from the tube with a spatula. 1.5 ml of TES and 0.6 ml ethidium bromide (10 mg ml^{-1}) was added to obtain a final density of 1.6 g ml^{-1}. The solution was centrifuged in self-sealing Beckman polypropylene tubes at 40,000 rpm for 40 hours at 18°C in a Beckman 50Ti rotor. DNA was visualised with a UV lamp and plasmid DNA was extracted by side-puncture. Ethidium bromide was removed from the DNA by extraction against butanol. The DNA was dialysed against three changes of TE buffer and then ethanol precipitated and reconstituted to a concentration of 1 mg ml^{-1}.

c. Lambda phage: Bacteriophage (10^5-10^6 plaque forming units; pfu) were adsorbed by incubation in 1 ml LM broth with E.coli LE392 cells (100 ul stationary phase culture grown in LM broth) at 37°C for 45 minutes. The cell suspension was then inoculated into 200 ml of LM broth and grown at 37°C with rapid shaking for 6 to 8 hours until lysis had occurred. The lysed culture was centrifuged at 8,000 rpm for 15 minutes to remove all cell debris and the supernatant digested with RNA'ase A (10 ug ml^{-1}) and DNA'ase I (10 ug ml^{-1}) at 37°C for 30 minutes. Bacteriophage were precipitated by the addition of sodium chloride to 0.5 M and PEG to 5%, and incubated overnight at 4°C. Phage were pelleted by centrifugation at 8,000 rpm for 20 minutes and the pellet resuspended in lambda

dilution buffer (200 mM NaCl, 20 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4) giving a titre of about 10^{12} pfu per ml. Phage DNA was extracted by the addition of SDS to 0.5%, EDTA to 50 mM, Proteinase K to 100 ug ml^{-1} and incubation at 65°C for one hour. Protein was solvent extracted from the DNA, the DNA ethanol precipitated and reconstituted in TE buffer.

d. Boiling method (Holmes and Quigley, 1981): Transformants were picked and grown overnight in 2 ml LB at 37°C with shaking. 1 ml of cells were centrifuged in Eppendorf tubes (Eppendorf centrifuge) for 30 secs. The pellet was resuspended in 200 ul of STET buffer (8 mg ml^{-1} glucose, 0.1 mg ml^{-1} Triton X100, 50 mM Tris-HCl, 50 mM EDTA, pH 8.0) and the solution boiled for 40 secs. The mixture was centrifuged for 15 minutes to pellet the cell debris and the supernatant removed to fresh tubes. DNA was precipitated twice, and redissolved in 20 ul of TE.

e. Alkaline lysis (Birnboim and Doly, 1979): 1.5 ml of an overnight culture of E.coli grown in LB medium was pelleted and resuspended in 50 mM glucose, 25 mM Tris-HCl pH 8 and 10 mM EDTA with lysozyme at 20 mg ml^{-1}. After the cells had been protoplasted they were lysed by the addition of 180 ul of cold 0.2 N NaOH, 1% SDS solution. After complete lysis 200 ul of 3 M potassium, 5 M acetate pH 4.8, was added. This solution made a flocculent white

precipitate between chromosomal debris bound with SDS and potassium. The precipitate was spun down, the supernatant removed and phenol-chloroform extracted. Ethanol, 1 ml, was added and after about 5 minutes at room temperature the DNA was collected by centrifugation. The pellet was washed in 70% ethanol, then dried in absolute ethanol. After removing the ethanol the DNA was redissolved in 50 ul of water. For a multicopy plasmid this procedure yielded about 2 ug.

2.2.7 Electrophoresis of nucleic acids

a. Eckhardt gels: Large plasmids in Rhizobium and Agrobacterium strains were visualised by using modifications of Eckhardt's original procedure (1978). The modification used early in this work was variably successful, and gave artifactual results. In this method approximately 10^8 early log-phase cells grown on TY medium at 30°C were washed once in TAE buffer and protoplasted in 50 ul of 1 mg ml^{-1} lysozyme, 100 ug ml^{-1} RNA'ase A, 160 mg ml^{-1} Ficoll-70 in TAE buffer, for 10 minutes at 37°C. Protoplasts were lysed with the addition of 50 ul of 0.02 mg ml^{-1} SDS and 160 mg ml^{-1} Ficoll-70 in TAE. After 5 minutes at 37°C, 10 ul of 5 mg ml^{-1} Proteinase K was added and digestion allowed for 10 minutes at 37°C. Lysates were then carefully loaded onto horizontal 0.6% agarose gels in TAE buffer and electrophoresed at 70mA for 5 minutes. The gel was then flooded with buffer to a depth

of 2 mm and electrophoresis continued at 45mA for 8 hours.

Later in this work a modification derived from the laboratory of A. Puhler became available. The gel was prepared with another set of wells, immediately behind the first. The second set of wells was filled with 1% SDS in 0.4% agarose and allowed to set. Exactly 10^8 early log-phase cells were protoplasted as before and loaded into the sample wells. Electrophoresis was started at 70mA and the SDS was electrophoresed through the protoplasts, causing cell lysis and liberating plasmid DNA into the gel. This method allowed the identification of very large megaplasmids which were only rarely seen by previous methods.

b. Agarose gels of restricted DNA: Horizontal slab gels of 140 x 160 x 4 mm dimensions made with 0.8% agarose (SeaKem, Marine Colloids, Inc.) in TAE buffer were routinely used for analysis of digested DNA. Samples were run with the addition of dye and heated at 65°C for 5 minutes prior to loading. Gels were run for 10 to 15 hours at 30 mA, 20V. All gel types were stained by soaking in 4 ug ml^{-1} ethidium bromide for 20 minutes followed by destaining in water for 20 minutes. DNA was visualised by exposure to ultraviolet light, 250 nanometers wavelength, using a transilluminator and photographed on Kodak type 107 or 667 film using a Kodak Wratten-gelatin filter No. 28. Molecular weight markers

were bacteriophage lambda cI857 Sam7 DNA cut with various enzymes.

c. Electroelution of DNA: Electroelution was used to purify particular DNA fragments of DNA. SeaPlaque (Marine Colloids Inc.) low gelling temperature agarose was always used and gels were run at 4°C. After staining to identify the fragments, gel slices were cut and placed in dialysis tubing. The tubing was filled with TAE buffer, sealed, placed in a gel tank and subjected to electrophoresis at 100mA, 80V for three hours. After this time the stained DNA could be seen outside the agarose. The solution in the tubing was removed and centrifuged to remove any small particles of agarose. The supernatant was phenol-chloroform extracted several times followed by chloroform extraction. DNA was ethanol precipitated and reconstituted in TE buffer at a concentration of 1 mg ml^{-1}.

2.2.8 Southern hybridisation procedures

a. Gel blotting: This method (Southern, 1975) was used to identify homologous DNA sequences in genomic or cloned DNA. Restricted DNA was resolved in agarose gels (Section 2.2.7 b.). If the DNA was of molecular size greater than 10 to 20 kb the gel was first soaked in 0.25 N HCl for 10 minutes to cause partial depurination of the DNA. This step was omitted for small molecules. Gels

were soaked in 0.5 m NaOH, 0.5 M NaCl for 30 minutes at room temperature to denature the double stranded DNA. The gel was then soaked for 30 minutes in 0.5 m Tris-HCl pH 7.2, 2 M NaCl to neutralise the base and to maintain denaturing conditions. The gel was then placed on filter paper wicks which were in contact with a solution of 20 x SSC. Wetted nitrocellulose was placed on the uppermost surface of the gel and two layers of wetted filter paper was placed on top. A wad of blotting paper was added to start blotting the denatured DNA onto the nitrocellulose. After about 12 hours the blotting paper had absorbed about 100 ml of the 20 x SSC. The nitrocellulose was removed, briefly washed in 2 x SSC, dried in the air, then baked *in vacuo* for 2 hours at 80°C. The nitrocellulose "filter" was then ready for hybridisation.

b. Radioactive probes: DNA probes were made by two methods.

Random priming: Plasmid DNA (1 ug) was restricted with the enzyme HaeIII and denatured by boiling for 2 minutes with 2 ug of random primers (8-12 nucleotide fraction of DNA'ase I treated calf thymus DNA) in 20 ul and cooled on ice for 2 minutes. Deoxynucleotides, dCTP, dGTP and dTTP (1 ul, 20 mM) and 3 ul of radioactively labelled dATP (3,000 Ci $mMol^{-1}$, 10 $mCi\ ml^{-1}$, BRESA) were added to the DNA and the reaction mix made up to 30 ul in HaeIII buffer. The sample was then incubated with 1 unit

of DNA polymerase I (large fragment) at 37°C for 30 minutes and the reaction stopped by phenol extraction. The phenol phase was washed twice with 50 ul of water and the combined fractions passed over a Sephadex G-50 column (bed volume 4 ml, medium grade G-50) with TES running buffer. Radioactively labelled DNA was monitored with a Geiger counter and eluted just after the void volume of the column (1 ml) whilst the unincorporated triphosphates were retarded on the column. The probe volume was usually about 600 ul.

Nick translation: 1 ug of DNA was made to a 20 ul volume in HaeIII buffer and 1 ul each of dGTP, dCTP and dTTP (all 20 mM) added. Radioactive dATP (1 to 3 ul), and about 1 unit of DNA polymerase I (Kornberg enzyme) was added with 1 ul of 10 ug ml^{-1} DNA'ase I and the mixture vortexed. After two hours of incubation at 16°C the reaction mixture was phenol extracted and run on the Sephadex G-50 column to separate incorporated from unincorporated radioactivity as described above. Both methods gave comparable incorporation of radioactivity into DNA. An average reaction would give about 10^6 to 10^7 counts per minute (cpm) per ug of DNA used.

c. Hybridisation conditions: Nitrocellulose filters were hybridised with probes in plastic bags. Firstly, the filter was wetted with pre-boiled hybridisation solution and pre-hybridised for one hour at 65°C. This procedure

tends to reduce non-specific binding of the probe to the filter. After pre-hybridisation, the hybridisation solution was reduced in volume to about 1 ml per 100 cm^2 of filter. The probe DNA was denatured by boiling for two minutes and then added to the hybridisation bag. The bag was sealed and the probe evenly distributed over the surface of the filter. The probe was allowed to hybridise with the filter at 65°C for varying times, usually 16 hours. After hybridisation, the filter was washed four times in 2 x SSC for about 30 minutes each time at room temperature. If too much non-specific binding of the probe had occurred the filter was re-washed at 65°C for one hour in 0.1 x SSC and 1% SDS. Filters were then dried in air prior to being exposed to X-ray film (Kodak XRP-5 and XAR-5 type film) at -80°C with Cronex (DuPont) "Lightning Plus" intensifying screens.

2.2.9 Cloning procedures

a. Dephosphorylation of vectors: Restricted DNA was dephosphorylated with calf intestinal alkaline phosphate (CAP), to prevent self-ligation. Restricted DNA was diluted to twice volume and pH to 10.4 with the addition of 0.1 volume of 1 M Tris buffer pH 10.4. 25 Units of CAP per ug of DNA was reacted for 5 minutes at 37°C. The reaction mixture was phenol-chloroform extracted. The DNA was ethanol precipitated, and redissolved in TE buffer.

b. Ligation: DNA was ligated in the presence of HaeIII or TA buffer with 0.5 mM ATP and 10^4 Units of T4 DNA ligase. Ligations were performed at 16°C overnight.

c. Transformation: Competent cells (prepared by the method of Morrison, 1979) were thawed on ice prior to transformation. Ligated DNA, diluted to a final volume of 100 ul, was added to 200 ul of thawed cells and left on ice for thirty minutes. The cells were heated in a water bath at 42°C for 2 minutes, and then inoculated into 5 mls of LB media (at 37°C). The solution was incubated at 37°C for one hour without shaking. Cells were concentrated by centrifugation, resuspended in 1 ml LB and plated onto appropriate selective antibiotic media.

2.2.10 Identification of recombinant plasmids

After plating on selective media, colonies were usually lifted onto nitrocellulose filters for hybridisation identification (Grunstein and Wallis, 1979) using a suitable probe. The filters were oriented to the pattern of colonies by stabbing the filter through to the agar with an inked needle. Filters were removed and placed, colony side up, on 3 MM (Whatman Ltd., England) paper, soaked with 0.5 M NaOH, 0.5 M NaCl for 5 minutes to lyse the cells and denature the DNA. Filters were then placed on 3 MM paper soaked with 0.5 M Tris-HCl pH 7.2, 2 M NaCl for 5 minutes. Filters were then washed in 2 x

SSC, dried and baked *in vacuo* for 2 hours at 80°C. The filters could then be hybridised to radioactive DNA probes as described in Section 2.2.8.c.

In some subcloning experiments insertional inactivation of vector antibiotic resistance genes could be used to identify recombinants. Usually 10 potential recombinants were screened directly for plasmid content to identify the desired plasmid.

2.2.11 Bacteriophage lambda bank

A bacteriophage lambda bank of *Rhizobium* strain ANU240 was constructed by J. Tellam. This was done by ligating size fractionated *Sau*3A-cut ANU240 DNA of 12-18 kb to replace the internal *Bam*HI "stuffer" fragment of the lambda vector Charon-28 (Liu *et al.*, 1980). The lambda bank was titred by plating for single plaques on lawns of *E.coli* LE392. This was done by mixing 60 *u*l of an overnight culture of LE392 with 100 *u*l of serial dilutions of the bacteriophage bank. The phage particles were allowed to adsorb at 37°C for 10 minutes before 3 ml soft agar was added and the culture plated onto LM agar plates. Plaques were scored after overnight incubation at 37°C. Bacteriophage preparations were stored in phage storage buffer (200 mM NaCl, 20 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4). Single pure plaques were picked, and placed in 1 ml phage storage buffer overnight. Bacteriophage were grown to high titre by infecting 10^8 cells of LE392 with about 10^5

pfu of bacteriophage and growing them in 5 ml LM liquid medium until lysis had occurred. The cell debris was pelleted and the supernatant stored over chloroform as a stock phage preparation.

For identification of recombinant bacteriophages by hybridisation to DNA probes, phage plaques were lifted onto nitrocellulose and treated exactly as bacterial colonies in Section 2.2.10.

Table 2.1 Bacterial strains used.

BACTERIAL STRAINS	RELEVANT CHARACTERISTICS	SOURCE OR REFERENCE
NGR234	Wild-type fast-growing cowpea *Rhizobium*.	Trinick, (1980)
ANU240	Sm^R NGR234 derivative.	This work
ANU239	Rif^R NGR234 derivative.	This work
ANU269	ANU239 carrying the plasmid pJB3JI.	This work
ANU264	A derivative of ANU240 which has lost the Sym plasmid and is Nod^-.	This work
ANU265	Sp^R ANU264 derivative.	This work
6015	*R.leguminosarum* strain, Nod^-. Plasmid deletion mutant which has lost symbiotic gene region. *phe*-1, *trp*-12, *rif*-392, *str*-37.	Johnston *et al*. 1978
ANU617	6015 carrying pJB5JI, Nod^+Fix^+ on peas.	B. Rolfe
ANU618	6015 carrying pBR1AN, Nod^+Fix^+ on clovers.	B. Rolfe
AK631	*R.meliloti* strain 41, Nod^+Fix^+ on lucerne.	A. Kondorosi

Table 2.1 continued. Important strains derived from NGR234.

Strain	Derived from:	Characteristics
ANU239	NGR234	Spontaneous Rif^R, Nod^+ Fix^+
ANU240	NGR234	Spontaneuos Sm^R, Nod^+ Fix^+
ANU264	ANU240	Heat-cured Nod^-, has lost the Sym plasmid, Sm^R.
ANU265	ANU264	Spontaneous Sp^R mutant, Nod^-.
ANU269	ANU239	Carries pJB3JI (Tc^R, Ap_R), Rif^R.
ANU271	ANU265	ANU265 (Sm^R, Sp^R) carrying pNM4AN, the cointegrate between pSUP1011 (Km^R, Cm^R) and the NGR234 Sym plasmid (Nod^+ Fix^+). Also has the helper plasmid pJB3JI (Tc^R). This strain can act as a donor to mobilise pNM4AN to other bacteria.
16-5	ANU240	Hac^- Nod^- mutant which has Tn*5* inserted in the Sym plasmid. Also called ANU1255.
ANU1256	16-5	Spontaneous Sp^R mutant.
U7	ANU240	Nod^+ Fix^- mutant which has Tn*5* inserted in the Sym plasmid. Also called ANU1245.
L10	ANU240	Defective nodulation mutant. Also called ANU1260.

Table 2.1 Continued.

ZB157	*R.meliloti* deletion mutant of AK631.	Banfalvi *et al.*, 1981
ANU1002	*R.trifolii* strain 5, Rif^R, Nod^+Fix^+ on clovers.	B. Rolfe
ANU1064	Heat-cured ANU1002, Nod^-.	B. Rolfe
ANU794	*R.trifolii* SU794, Nod^+Fix^+ on clovers.	J. Vincent
ANU843	*R.trifolii* SU843, Nod^+Fix^+ on clovers.	J. Vincent
ANU845	Heat-cured, Nod^-, ANU843.	M. Djordjevic
ANU453	Tn*5*-induced Hac^-Nod^- mutant of ANU794.	B. Rolfe
ANU851	Tn*5*-induced Hac^-Nod^- mutant of ANU843.	M. Djordjevic
C58	Virulent *Agrobacterium tumefaciens* strain.	J. Schell Van Larebeke *et al.*, 1974
A136	Avirulent cured strain of *A.tumefaciens* C58.	P. Hooykaas
RR1	*E.coli* strain, F^-, *hsd*S20 (r^-,m^-), *ara*-14, *pro*A2, *gal*K2, *rps*L20, *xyl*-5, *mtl*-1, *sup*E44, *lac*Y1, $lambda^-$.	Bolivar *et al.*, 1977

Table 2.1. Continued

LE392	*E.coli* strain, *hsd*R514 (r^-,m^-), *sup*E44, *sup*F58, *lac*Y1, *gal*K2, *gal*T22, *met*B1, *trp*R55, lambda$^-$.	Grunstein and Wallis, 1979
SM10	*E.coli* strain, used for mobilising plasmids. Has an Ap^S, Tc^S, Km^R, RP4::Mu integrated in the chromosome. Can mobilise plasmids *in trans*.	Simon *et al*., 1983

Table 2.2 Plasmids and vectors used in this study.

VECTOR	CHARACTERISTICS	SOURCE OR REFERENCE
RP4	IncP1, Tra^+, Km^R, Tc^R, Ap^R. Stably maintained in *Rhizobium*.	B. Holloway Datta *et al.*, 1971
R68.45	IncP1, Tra^+, Km^R, Tc^R, Ap^R.	B. Holloway
pJB3JI	A derivative of R68.45 which does not confer Km^R.	Brewin *et al.*, 1980b
pJB4JI	IncP1, Tra^+, pPH1JI::Mu::Tn*5*, Km^R, Gentamycin resistant pPH1 was derived from the plasmid R751. Suicide mutagenesis plasmid.	Beringer *et al.*, 1978a
pJB5JI	*R.leguminosarum* Sym plasmid which contains Tn*5*. Self-transmissible.	Brewin *et al.*, 1980b
pBR1AN	*R.trifolii* Sym plasmid derived through recombination between pJB5JI and a *R.trifolii* Sym plasmid. Self-transmissible.	Djordjevic *et al.*,1983

Table 2.2 Continued.

pSP601	IncP1, Tra^+, R751 derivative which carries Tn5 (Km^R), Tn1771 (Tc^R), Tn1 (Ap^R) and Mu. Suicide mutagenesis plasmid.	A. Puhler
RP4::MuCts ::Tn7	RP4 carrying MuCts and Tn7, confers Sm^R and trimethoprim resistance in E.coli. MuCts is a thermo-inducible mutant in the Mu phage C gene. Retains all markers of RP4.	J. Beringer
pRK2013	This plasmid has the Tra genes and ori-T of the IncP1 plasmid RK2 cloned onto a ColE1 derivative. RK2 is indistinguishable from RP4. This plasmid can transfer to Rhizobium but cannot be maintained.	Figurski and Helinski, 1979.
pRmSL26	A recombinant plamid with a 20kb section of the R.meliloti strain 1021 megaplasmid. Insert contains the Hac region.	Long et al., 1982
pRD1	R-prime plasmid which contains the entire Klebsiella pneunomiae nif operon. Plasmid was derived from RP4.	Dixon and Postgate (1971)

Table 2.2 Continued.

pRme41b:pAK11	A mobilisable *R.meliloti* megaplasmid, formed by by recombination in the *nif* H gene. Cannot confer Fix^+.	Kondorosi *et al.*, 1983
pSUP101	pACYC184 derivative which has the ori-T or Mob of RP4 cloned into the *Bam*HI site. Confers Cm^R in *E.coli*. Can be mobilised *in trans* by *Inc*P1 transfer proficient plasmids.	Simon *et al.*, 1982
pSUP1011	pSUP101::Tn*5* suicide mutagenesis vector. Confers Cm^R and Km^R in *E.coli*. Can be mobilised *in trans* as for pSUP101. Cannot replicate in *Rhizobium*.	Simon *et al.*, 1982
pACYC177	*E.coli* specific cloning vector Ap^R, Km^R.	Chang and Cohen, 1978
pSUP106	Plasmid capable of replicating in *Rhizobium* derived from the *Inc*Q group plasmid RSF1010. *E.coli*-*Rhizobium* shuttle vector. Cm^R, Tc^R, Mob^+.	Simon *et al.*, 1983

Table 2.2 Continued.

pSUP202	pBR325 derivative carrying Mob site. Cm^R, Ap^R, Tc^R. Can be mobilised *in trans* but cannot replicate in *Rhizobium*.	Simon *et al.*, 1983
pNAM2	Similar construct to pSUP106 but with Km^R, Ap^R.	This work
pUC8	*E.coli* specific cloning vector, Ap^R.	Vieira and Messing, 1982
pBR322	*E.coli* specific cloning vector, Ap^R,Tc^R.	Bolivar *et al.*,1977
pBR328	Ap^R, Tc^R, Cm^R *E.coli* specific cloning vector.	Soberon *et al.*, 1980
pKan2	Specific Tn*5* probe. Contains internal 3.5kb *Hin*dIII fragment of Tn*5* cloned into pBR322.	Scott *et al.*, 1982
pRtnif-2	3.5kb clone of *R.trifolii* *nif*HD gene region.	Scott *et al.*, 1983b
pRtO32	14kb clone of *R.trifolii* Nod gene region, cloned on pKT240.	Schofield *et al.*, 1983b

Table 2.3 Plasmid constructions made during this thesis.

PLASMID	INSERT	PARENT VECTOR
p16-5	Tn*5*-containing *Eco*RI fragment from mutant 16-5	pBR328
pU7	Tn*5*-containing *Eco*RI fragment from mutant U7	pBR328
pB7-42	Tn*5*-containing *Eco*RI fragment from mutant B7-42	pBR328
pC5-25	Tn*5*-containing *Eco*RI fragment from mutant C5-25	pBR328
pRs1	2.3 and 6.7kb *Hin*dIII fragments from NGR234 Nod region	pBR322
pRs2	24kb *Hin*dIII fragment which hybridises to a repeated sequence in pRs5 and lambda 20	pBR322
pRs3	9.4kb *Hin*dIII fragment	pBR322
pRs5	9.4kb *Hin*dIII fragment from lambda 20	pNAM2
pRs7	6kb *Eco*RI fragment from lambda 20	pNAM2
pRs15	8kb *Eco*RI fragment from lambda 20	pNAM2
pRs17	8kb *Bam*HI fragment from lambda 20	pNAM2
pRs19	6.7kb *Hin*dIII fragment	pBR322
pRs20	2.3kb *Hin*dIII fragment	pBR322
pRs21	6.7kb *Hin*dIII fragment	pSUP106
pRs23	6.7kb *Hin*dIII fragment	pSUP202

Table 2.3 Continued.

pRs24	2.3kb HindIII fragment	pSUP106
pRs25	4.5kb XhoI-HindIII fragment from pRs5	pSUP202
pRs50	16kb XhoI fragment	pACYC177
pRs51	16kb XhoI fragment	pSUP106
pRs52	2.8kb EcoRI fragment	pUC8
pRs53	1.4kb BamHI fragment	pUC8
pRs54	1.7kb BamHI fragment	pUC8

Table 2.4 Plant species and cultivars.

PLANT SPECIES	COMMON NAME AND CULTIVAR
Macroptilium atropurpureum	cv. siratro
Macroptilium lathyroides	Phasey bean
Lablab purpureus	Lablab cv.Highworth
Vigna unguiculata	Cowpea cv. Poona
Desmodium intortum	Greenleaf desmodium
Desmodium uncinatum	Silverleaf desmodium
Stylosanthes hamata	cv. Verano
Leucaena leucocephala	cv. Peru
Glycine max	soybean cv. Bragg
Glycine tomentella	
Glycine tabacina	
Glycine wightii	cv. Caeserii
Macrotyloma axillaris	
Trifolium repens	White clover cv. NZ 5826 "Hivi"
Trifolium subterraneum	Subterranean clover cv. Mt. Barker
Medicago sativa	Lucerne cv. Hunter valley
Pisum sativa	Pea cv. Greenfeast
Parasponia andersonii	

Sources: All seeds except Parasponia andersonii, Glycine tomentella and G.tabacina were obtained from J.H. Williams and Sons, Seed Merchants, Murwillumbah, Australia. P.andersonii was obtained from Dr. M.J. Trinick, C.S.I.R.O., Division of Land Resource Management, Perth. Glycine species were obtained from Dr. Adrian Gibbs, Virus Ecology Research Group, Research School of Biological Sciences, Australian National University, Canberra.

CHAPTER THREE

GENERAL CHARACTERISTICS OF STRAIN NGR234

3.1 INTRODUCTION

The purpose of this work was to describe salient characteristics of the fast-growing cowpea strain NGR234 which were pertinent to genetic and symbiotic studies. The host-range of this organism was already known to be similar to that of slow-growing cowpea *Rhizobium* strains, particularly CP283, therefore the comparison with slow-growing rhizobia depends mainly on the utilisation of carbon compounds and natural tolerance to antibiotics. The recently identified fast-growing *R.japonicum* strains from China (Keyser *et al.*, 1981) are compared with NGR234 with respect to host range to see if these strains also have the broad-host-range characteristic of NGR234 or whether they behave more like slow-growing *R.japonicum* strains. The plasmid content of these strains is also investigated.

3.2 INITIAL PURIFICATION OF STRAIN NGR234

Despite the purification of NGR234 by Trinick (1980) the strain had at times been suggested to be contaminated with a slow-growing cowpea Rhizobium organism which was responsible for nodulation, and the fast-growing organism was in fact a non-nodulating Agrobacterium radiobacter strain of some kind which was able to coinfect with the slow-grower. To ensure that work was started with a pure strain, a culture of NGR234 was purified to single colonies by serial dilution. The detergent Tween-80 was used at 0.1% in the dilution fluid since microscopic examination showed that vortexing in this solution produced a uniform suspension of single cells. This was done to ensure that colonies arose from single cells, thereby separating potential contaminants. The single colonies which arose were all uniform in appearance and the viable counts corresponded with estimates from haemocytometer counts. Since slow-growing rhizobia are notoriously "sticky" it was still possible that the culture was contaminated, so the entire procedure of selecting a colony, diluting in 0.1% Tween-80, and plating for single colonies was repeated three consecutive times. Finally a single pure colony was chosen for all subsequent studies. This clone of NGR234 was inoculated on siratro and subsequently reisolated from nodules. The ex-nodule isolate had all the cultural characteristics of the

inoculum. The clone of NGR234 was found to effectively nodulate Lablab purpureus and cowpea. Later the same clone was shown to nodulate Leucaena leucocephala and Parasponia andersonii. Ex-nodule isolates from cowpea and Leucaena had all the characteristics of the inoculum strain. No change in nodulation host-range occurred as a result of passage through either siratro, cowpea or Leucaena, showing that the broad-host-range characteristic is stable.

The closest relatives to strain NGR234 are the fast-growing Leucaena isolates described by Trinick (1980) some of which can nodulate siratro, however none of these can produce normal, nitrogen-fixing nodules on Lablab purpureus. The effective symbiosis between NGR234 and Lablab purpureus constitutes proof of strain identity.

3.3 GROWTH ON LABORATORY MEDIA

Strain NGR234 can grow on the Rhizobium growth media normally used for R.trifolii (BMM, TM, TMY, TY, and YM) but cannot grow on LBG media (for E.coli, R.meliloti and A.tumefaciens), or any media supplemented with high salt concentrations. Colonies on TY media are beige coloured, non mucoid, low, flat, circular, entire edged and produce a faint green pigment which diffuses into the agar. After 3 days at 30°C colonies are about 2 mm in diameter. A slow-growing strain will usually produce colonies of only

0.1 mm after this time. On BMM strain NGR234 colonies are white, glistening and slightly mucoid but not gummy or sticky. On TMY medium the colonies are yellowish and less mucoid than on BMM.

NGR234 will not grow on any tested media at temperatures above 35°C. Prolonged exposure to elevated temperatures (37 or 43°C) results in loss of viability. In contrast, slow-growing rhizobia can tolerate prolonged exposure to high temperatures with little loss of viability when returned to growth-permissive temperatures (G. Bender, personal communication). This difference was used to check again for some kind of slow-growing contaminant in NGR234 by looking for slow-growing survivors of high temperature treatment.

NGR234 was tested for sensitivity to various antibiotics. Minimal inhibitory concentrations (in ug ml^{-1}) were uniformly low: Km, 50; Sm, 50; Tc, 2; Ap, 50; Rif, 10; Cm, 5, $HgCl_2$, 5. In contrast, slow-growing cowpea rhizobia (like CP283 and CB756) are resistant to unusually high levels of Cm, Rif and Tc (G. Bender, personal communication). This again emphasises that NGR234 is not contaminated by a slow-growing cowpea *Rhizobium* strain since such a contaminant would have become evident during these antibiotic sensitivity tests.

3.4 COMPARISON OF STRAIN NGR234 WITH SLOW-GROWING COWPEA *RHIZOBIUM* STRAINS WITH RESPECT TO UTILISATION OF VARIOUS CARBON SOURCES

Results are presented in Table 3.1. Data presented for slow-growing strains were done entirely by Mr. G. Bender.

3.4.1 Organic acids: Good growth occurred on succinate, fumarate, malate and pyruvate. On malate the colonies were raised gummy and white, while on succinate and fumarate the colonies were very raised and ochre in colour. On pyruvate the colonies were flat and white. These colony differences apparently reflect different metabolic states of the bacteria. Neither acetate or citrate were able to support growth, whilst oxaloacetate, glyoxalate and malonate allowed colonies to grow at a slow rate. Colonies on glyoxalate were low, flat and watery.

When D-glutamate was used as a nitrogen source instead of the ammonium ion, the final results were very similar except that the onset of growth on succinate or fumarate was delayed by about three days. This effect was less apparent with malate.

The slow-growing strains CB756 and CP283 were completely inhibited on media containing 50 mM succinate. In contrast a steady increase in growth was found with NGR234 on succinate concentrations from 10 mM to 100 mM.

3.4.2 Sugars: NGR234 grew well only on D-gluconate, D-glucose, sucrose and D-mannose (in order of best growth). Two colony forms appeared on D-gluconate; one being large, raised and gummy which grew rapidly and was visible after two days, the other being small, white, flat and entire edged. The large colony form occurred about once per 500 small colonies. The large colonies fluoresced green under ultraviolet light of 350 nm, while the small form did not. In comparison, all colonies grown on succinate media fluoresced orange under 350 nm light. Colonies on normal TM did not fluoresce.

3.5 COMPARISON BETWEEN NGR234 AND FAST-GROWING *R.JAPONICUM* STRAINS FROM CHINA

3.5.1 Symbiotic host-range: A selection of fast-growing *R.japonicum* strains and NGR234 were tested for symbiosis with a range of large and small seeded legumes using Leonard jar and test tube assays. The results are shown in Table 3.2. Controls included an equal number (5) of uninoculated tests and tests watered with 1 mM nitrate nutrient medium. Strain CP283 was also included as a control for nodulation of the cowpea plants.

The results show that the symbiotic host-ranges of NGR234 and the fast-growing *R.japonicum* strains overlap considerably. The only discriminating plants are *Lablab*

purpureus, Desmodium intortum and Glycine tabacina. Slow-growing R.japonicum strains are reported to vary in ability to effectively nodulate in the cowpea group of plants (Allen and Allen, 1981) and the reverse situation is also true, that some cowpea Rhizobium strains can nodulate soybean. The criterion of classification of these fast-growing strains as R.japonicum, derives from their ability to symbiose effectively with the ancestral soybean (Glycine soja) and the Peking variety of soybean (Keyser et al., 1981). Since NGR234 nodulates soybean (Glycine max) it could also be thought as a R.japonicum strain. However, the NGR234 host-range certainly encompasses more plants than those normally nodulated by a R.japonicum strain. NGR234 can nodulate Leucaena leucocephala and Acacia farnesiana (Trinick, 1980), plants which are in a different sub-family to the soybean or cowpea. Keyser et al. (1981) reported that the fast-growing R.japonicum strains cannot nodulate Leucaena leucocephala. Even CP283, the broad-host-range slow-growing cowpea strain, does not nodulate Leucaena.

3.5.2 Plasmid content: The plasmids of the fast-growing R.japonicum strains were investigated in conjunction with Mr. Y.H. Cen and Dr. J. Plazinski. These results are essentially the same as those of Appelbaum and Chartrain (1983) and are shown in Figure 3.1. All fast-growing R.japonicum strains harboured very large plasmids of

various sizes and all contained megaplasmids. Hybridisations using a "Nod" gene probe from NGR234 (described in Chapter Seven) showed that certain of the smaller plasmids contained nodulation gene sequences and were probably Sym plasmids. Heat-curing (as described in Chapter Four) of a single plasmid in USDA205 lead to a loss in nodulation ability. In numerous attempts by Mr. Y.H. Cen and Dr. J. Plazinski no evidence for plasmids of any kind could be found in slow-growing cowpea or Parasponia-nodulating Rhizobium strains. The plasmids of NGR234 are described in Chapter Four.

3.6 DISCUSSION

The fast-growing cowpea strain NGR234 is physiologically and culturally dissimilar to the slow-growing cowpea Rhizobium strain CP283, which has a very similar host-range. In its behaviour on laboratory media, NGR234 is like R.trifolii and R.leguminosarum strains but not like R.meliloti strains. The amount of exopolysaccharide produced on mannitol medium is not excessive, and on TY medium very little exopolysaccharide and no gum is produced. These characteristics are shared with the fast-growing R.japonicum strains.

Innate resistance of NGR234 to antibiotics is also similar to other fast-growing strains. Slow-growing cowpea and R.japonicum strains are reported to be

resistant to high levels of most antibiotics (Trinick et al., 1983). A strong positive correlation exists between resistance to rifampicin and the ability to derepress the nitrogenase enzyme complex in vitro (Pankhurst et al., 1982). NGR234 is sensitive to low levels of rifampicin and has not been conclusively shown to be able to derepress nitrogenase (G. Bender, personal communication). NGR234 is also sensitive to the lysing action of mild detergents and lyzosyme while the slow-growing cowpea strains CP283 and CB756 are resistant to prolonged exposure to lysing agents (J. Plazinski, personal communication).

The analysis of plasmids shows that NGR234 and the fast-growing R.japonicum strains have an arrangement of plasmids similar to that found in other fast-growing rhizobia. This arrangement consists of a megaplasmid and a variable number of smaller plasmids, one of which may be a Sym plasmid. This is in sharp contrast to the slow-growing R.japonicum and cowpea Rhizobium strains where no Sym or megaplasmids have been identified.

In conclusion, strain NGR234 has a nodulation host range which includes the Leucaena-Acacia inoculation group, the "cowpea" miscellany and the soybean inoculation group. Its most effective symbioses are within the cowpea group, yet NGR234 is quite distinct in many characteristics from the slow-growing cowpea rhizobia. The closest relatives to NGR234 are the Leucaena rhizobia

described by Trinick (1980) and the fast-growing R.japonicum species. In both cases, the ability of NGR234 to form an effective symbiosis with Lablab purpureus sets it apart from these other strains. NGR234 appears to be the best Rhizobium strain in which to investigate the control of the broad-host-range nodulation ability of cowpea rhizobia.

SUMMARY

1. NGR234 is only superficially similar to the slow-growing cowpea *Rhizobium* strains CP283 and CB756, and the *Parasponia Rhizobium* strain NGR231.

2. NGR234 is not contaminated by a slow-growing cowpea *Rhizobium*.

3. Fast-growing *R.japonicum* strains from China are culturally similar to NGR234, have a similar content of plasmids, and share certain host specificities, including effective nodulation of cowpeas and ineffective nodulation of soybeans.

4. NGR234 has a wider host range than the fast-growing *R.japonicum* strains, and is therefore a cowpea *Rhizobium* rather than a *R.japonicum* .

Table 3.1 Growth of fast- and slow-growing *Rhizobium* strains on various carbon sources; average colony diameter after 3 or 10 days.

Carbon source	STRAIN NGR234	CP283	CB756	NGR231
Time: days	3	10	10	10
1. *Control media*				
TMY	2.0	0.2	0.4	0.4
TM- no carbon	0.1	<0.1	<0.1	<0.1
2. *Organic acids*				
Succinate	2.0	0.1	0.3	0.5
Fumarate	1.5	0.1	0.4	0.5
Pyruvate	2.0	0.2	0.2	0.3
Citrate	-	0.2	0.2	0.5
Acetate	-	0.1	0.2	-
Malate	1.5	0.2	0.2	0.5
Oxaloacetate	1.0	-	-	-
Glyoxalate	1.0	0.2	0.2	-
Malonate	1.0	0.1	<0.1	0.4
D-Gluconate	1.0	0.3	0.5	0.8

Table 3.1 Continued.

3. Sugars				
D-Glucose	0.4	0.2	0.1	0.3
D-Mannose	0.4	0.3	0.1	0.6
D-Fructose	-	0.2	0.2	0.4
D-Galactose	-	0.2	0.2	0.4
D-Arabinose	-	0.3	0.3	0.3
L-Arabinose	-	0.3	1.0	0.7
D-Xylose	-	0.3	0.2	0.8
D-Mannitol	-	0.2	0.5	0.6
D-Glycerol	-	0.2	0.1	0.3
D-Inositol	-	-	-	0.2
D-Sorbitol	-	0.2	0.2	0.1
D-Sucrose	0.4	-	0.1	0.2

The basic medium used was TM- no carbon, which is TM minimal medium with no carbon source, no vitamins and 5mM ammonium nitrate as a nitrogen source. Organic acids were at 10mM and sugars were at 50mM. Media was set initially at pH 7.0.

Results presented for strains CP283, CB756 and NGR231 were the work of Mr. G. Bender, as mentioned in the text.

Table 3.2 Nodulation and nitrogen fixation of various plants inoculated with fast-growing *Rhizobium japonicum* strains and fast- and slow-growing cowpea *Rhizobium* strains.

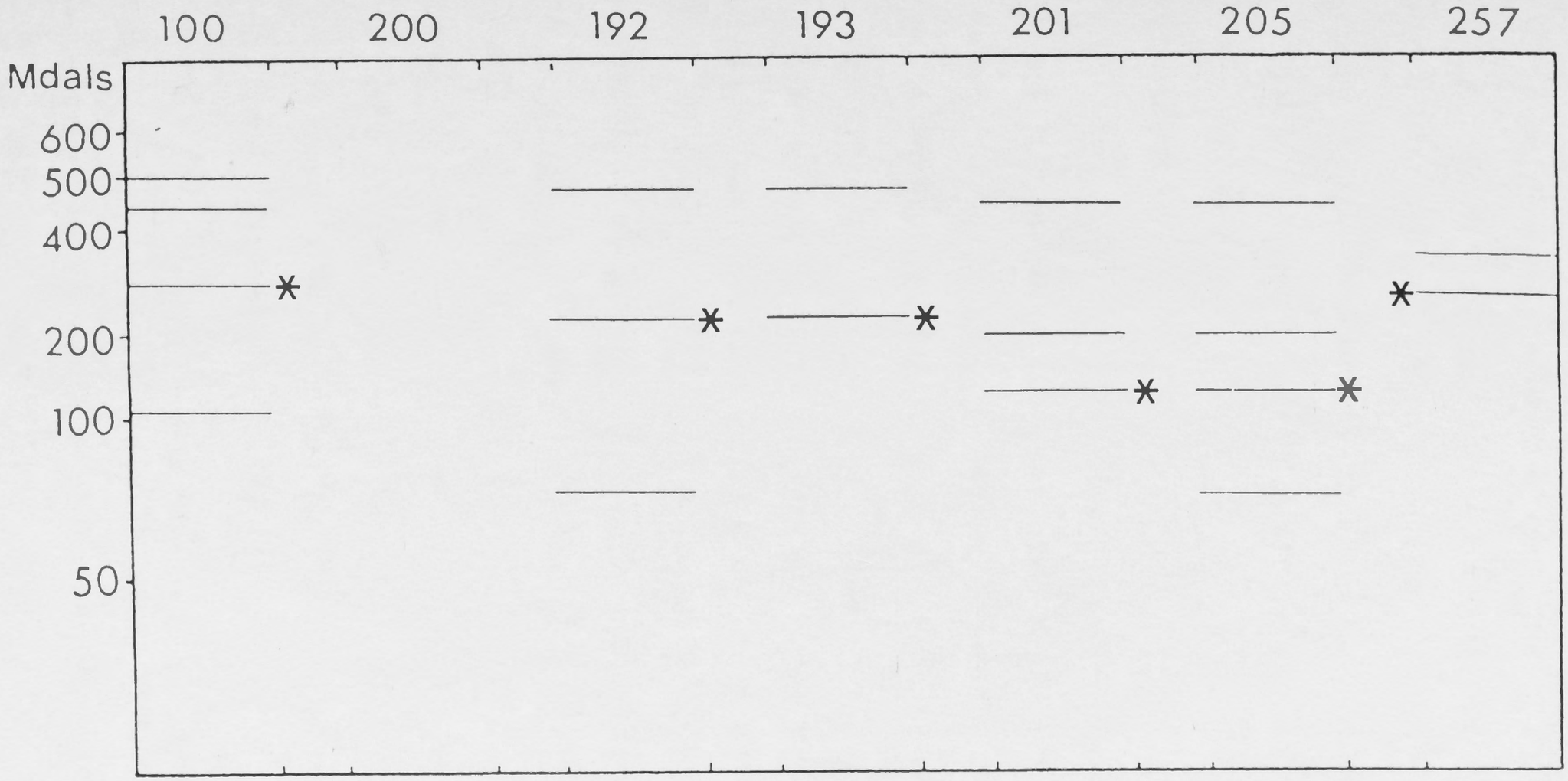
PLANT USED	STRAINS:	CP283		ANU240		USDA192		USDA193		USDA201		USDA205		USDA257	
	RESPONSE:	N	F	N	F	N	F	N	F	N	F	N	F	N	F
1. Plants cultivated in Leonard jars:															
Cowpea		+	E	+	E	+	E	+	e	+	E	+	e	+	E
Soybean		-	-	+	I	+	I	+	I	+	I	+	I	+	I
Siratro		+	E	+	E	+	I	+	E	+	I	+	I	+	I
Glycine whigtii		+	E	+	E	+	e	+	I	+	E	+	I	+	I
Lablab purpureus		+	E	+	E	-	-	-	-	-	-	-	-	-	-
2. Plants grown in tubes:															
Siratro		+	E	+	E	+	e	+	E	+	I	+	I	+	I
Phasey bean		+	E	+	e	-	-	+	I	+	I	-	-	+	I
Teramnus uncinatus		+	E	+	e	+	I	N.T.		+	I	+	I	+	I
Glycine tabacina		+	e	+	I	-	-	-	-	-	-	-	-	-	-
Glycine tomentella		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Desmodium intortum		+	E	+	E	-	-	-	-	-	-	-	-	-	-
Desmodium uncinatum		+	E	+	e	-	-	-	-	-	-	-	-	-	-
Macrotyloma axillaris		+	E	-	-	-	-	-	-	-	-	-	-	-	-

Note: N refers to nodulation. F refers to effectiveness of symbiosis; E means effective, e means partially effective, I means ineffective. N.T. means not tested.

This work was done under the supervision and with the assistance of Dr. M.J. Trinick in his laboratory in Perth.

Figure 3.1. Diagrammatic representation of plasmids in fast-growing R.japonicum strains representing cumulative data of Appelbaum and Chartrain (1983), Appelbaum et al. (1984), Mr. Y.H. Cen and Dr. J. Plazinski (personal communication). This diagram serves to illustrate how NGR234 is similar to the fast-growing R.japonicum strains in plasmid content, both having cryptic megaplasmids and smaller Sym plasmids. Plasmids which hybridise to either Nod or nif probes are asterisked; this information was derived from all the quoted authors. The Sym plasmid in USDA205 was identified by heat-curing. The numbers refer to USDA strains, with the exception of 100 and 200 which refer to ANU100 (also known as IHP-100, a fast-growing cowpea Rhizobium strain from India), and ANU200 (also known as ORS-571, a Rhizobium strain from Sesbania rostrata, which is able to form stem nodules). The data on these two strains was the work of Dr. J. Plazinski, who also provided this diagram.

The plasmids of molecular weight greater than 400 Mdals (megaplasmids) in the fast-growing R.japonicum strains were identified by Dr. J. Plazinski. Smaller plasmids were identified by the author.


100
200
192
193
201
205
257
Mdals
600
500
400
200
100
50

CHAPTER FOUR

A LARGE PLASMID IN NGR234 CARRIES GENES FOR NODULATION AND NITROGEN FIXATION

4.1 INTRODUCTION

It has been shown that the nodulation properties of many fast-growing R.trifolii strains can be lost through a process of prolonged exposure to elevated temperatures. The eradication of the symbiotic capability is usually the result of a total loss of certain large endogenous plasmids or deletions in these plasmids (Zurkowski and Lorkiewicz, 1978). As well as R.trifolii, heat-curing has been successful in R.leguminosarum (Sanders et al., 1978) and A.tumefaciens. When applied to R.meliloti the heat-curing technique has yielded deletion mutants of the R.meliloti megaplasmid but has not lead to the complete loss of this plasmid (Banfalvi et al., 1981). The lack of literature reports on heat-curing the symbiotic ability of slow-growing Rhizobium species and the lack of success with slow-growing Parasponia Rhizobium species (Y-H. Cen, personal communication) suggests that slow-growing rhizobia are resistant to this treatment. This chapter describes the heat-curing of a large plasmid in strain NGR234 and proof that this plasmid is required for both

nodulation and nitrogen fixation and is indeed a Sym plasmid. Further work describes the behaviour of the exogenous Sym plasmids pJB5JI (R.leguminosarum) and pBR1AN (R.trifolii) in NGR234 derivatives, with respect to nodulation and nitrogen fixation.

4.2 HEAT-CURING AND PLANT TESTS

Two loopsful (approximately 10^{10} cells) from a culture of ANU240 grown on TY medium for 3 days, were evenly spread on the surface of solid YM medium. The plates were sealed with Nescofilm except for a small hole, and then incubated at 37°C for 7 days, during which time no growth occurred. The plates were then placed at room temperature and after 5 days, approximately 100 single colonies arose on each plate. No inhibition of growth was noted in cultures prepared similarly and incubated at 30°C and 35°C. About 100 single colonies were purified from these plates incubated at 37°C. These strains all had the characteristic colony morphology of strain NGR234 when grown on TY medium and all were Sm^R. A total of 30 pure clones were tested for nodulation capabilities on siratro, of these 7 failed to nodulate.

The inoculum strain was recovered from the plant test agar in the 7 non-nodulating strains, and each was purified to single colonies. Again all had the characteristics of strain ANU240. The reisolated strains

were retested for nodulation of siratro using 6 plants per test. The nodulating and non-nodulating phenotypes were found to be stable and non-reverting through subculture. Eckhardt plasmid gels showed that the nodulating strains all had the NGR234 plasmid, whilst the non-nodulating strains had all lost this plasmid. Since the seven Nod^- strains appeared identical, one strain, designated ANU264, was chosen for further study. A plasmid gel analysis of this strain, compared with the wild-type strain ANU240 (Fig.4.1), shows that both strains retain a large megaplasmid, of unknown size, while strain ANU264 has definitely lost the smaller plasmid.

As well as being Nod^- on siratro, strain ANU264 was unable to nodulate *Desmodium intortum*, *Vigna unguiculata*, *Lablab purpureus*, *Macroptilium lathyroides*, *Leucaena leucocephala* and *Parasponia andersonii*. Strain ANU240 and a Nod^+ strain from the heat-curing experiments were able to nodulate all these plants. To determine the stage at which the process of nodulation is blocked in the heat-cured strain ANU264, the slide method of Fahraeus (1957) was used to microscopically examine the roots of siratro plants inoculated with either strain ANU240 or ANU264. While strain ANU240 was able to induce curling, extreme distortions and infection threads on root hairs, the mutant strain ANU264 had no observable effect on root hair morphology (Fig. 4.2).

4.3 HYBRIDISATION TO A *NIF* PROBE

A cloned DNA fragment (pRtnif-2) containing the entire *nifH* gene and 141 codons of the *nifD* gene from the *R.trifolii* strain SU329 (Scott *et al.*, 1983b) was used as a hybridisation probe in Southern analysis to determine whether the structural nitrogenase genes are present on the NGR234 plasmid. The method used was to hybridise this probe on to restriction endonuclease-treated total DNA from strain ANU240 and strain ANU264. DNA from strains ANU264 (pJB5JI) and ANU264 (pBR1AN) (strains described later) were included as controls since both these plasmids are known to contain nitrogenase genes of known restriction pattern. This experiment showed that the *nif* probe hybridised to DNA of strains ANU240, ANU264 (pJB5JI) and ANU264 (pBR1AN) but the probe did not hybridise to ANU264 (Fig. 4.3). The *nifH* and *nifD* gene region of strain ANU240 is contained on two *EcoRI* fragments of 3.2 and 4.0kb. The *nifH*-and *nifD*-specific hybridisation in strains ANU264 (pJB5JI) and ANU264 (pBR1AN) is characteristic of the *nif* gene regions of these two introduced plasmids and not of strain ANU240. The hybridisation is strongest to the DNA of strain ANU264 (pBR1AN) while the *nif* regions identified in ANU240 and those in ANU264 (pJB5JI) hybridise at approximately equal intensity.

The difference in hybridisation intensity may be

caused by certain regions of the probe fragment being represented by homologous regions in R.trifolii (and therefore pBR1AN) and not in the other strains. It is known that this probe fragment contains a R.trifolii-specific repeated sequence in the nifH promoter region and also contains about 1.5kb of DNA of unknown function on the 5' side of the nifH gene (Scott et al., 1983b). This DNA may be conserved in R.trifolii strains but may not be present in other Rhizobium strains. It is also possible that the variation in hybridisation intensity is due to slight divergence of the nifH and nifD sequences. This hybridisation was done under stringent conditions and rather small deviations in sequence homology may lead to large changes in hybridisation intensity. If this is true it predicts that the nifH nifD zone of ANU240 is about as evolutionarily removed from R.trifolii as is the R.leguminosarum nifH nifD zone identified in strain ANU264 (pJB5JI). The answer to this question must await DNA sequencing of the nif genes of strain ANU240.

4.4 REINTRODUCTION OF THE NGR234 SYM PLASMID TO THE HEAT-CURED STRAIN

To prove that the plasmid lost in ANU264 is indeed a Sym plasmid it was necessary to reintroduce the plasmid to the heat-cured strain. This was achieved by using the IncP1 group plasmid RP1::Tn501 to mobilise the plasmid

from ANU239 (a Rif^R mutant of NGR234) to strain ANU265 (a Sp^R mutant of ANU264). These two strains were constructed to provide adequate markers for identification and contra-selection.

The plasmid RP1::Tn*501* was transferred from *E.coli* to ANU239 at a frequency of 10^{-2}. A single transconjugant was purified and used as a donor strain in a filter cross with strain ANU265. Total cells were diluted and plated on TY medium containing 250 ug ml^{-1} each of streptomycin and spectinomycin (to select for ANU265) and 200 ug ml^{-1} of kanamycin (to select for the RP1::Tn*501*). The frequency of transfer was 10^{-2}. Large numbers of transconjugant clones were purified, *en masse*, by replica plating on selective media through two subcultures. Total cells were washed off the selective plates, and appropriate dilutions were used to inoculate siratro plants. These dilutions were also plated onto Rif-containing medium to check for the presence of the donor strain. A few Rif^R colonies arose, but when purified they all failed to nodulate siratro and probably were spontaneous mutants of ANU265, as they were also Sm^R and Sp^R.

In contrast to the control ANU239 (RP1::Tn*501*) which nodulated well at low cell numbers, nodules arose (38 in number) only on plants inoculated with high cell numbers of total ANU265-selected cells from the mating. The appearance of nodules was also delayed in comparison with

control experiments done with ANU239 (RP1::Tn*501*). The former took three weeks for the first appearance of nodules, whereas the latter gave good nodulation within 10 days. This probably indicates that the number of cells capable of nodulation was low. Bacteria were reisolated from all 38 nodules and tested for their antibiotic resistances. All were Sm^R and Sp^R, like ANU265, and none were Rif^R. Thirteen Nod^+ ANU265 transconjugants were assayed for plasmid content by the Eckhardt technique. All 13 strains contained a plasmid band of the same size as RP1::Tn*501* and a plasmid the same size as the NGR234 Sym plasmid (Fig. 4.4). Two strains showed a Fix^+ response on retesting with siratro.

The same type of experiment as described above was done using the plasmid R68.45 and using strain ANU239 alone. Neither experiment demonstrated transfer of nodulation ability to strain ANU265.

4.5 TRANSFER OF THE SYM PLASMIDS pJB5JI AND pBR1AN TO WILD-TYPE AND HEAT-CURED NGR234 DERIVATIVES

The symbiotic host-range and nitrogen fixation ability of the *R.leguminosarum* Sym plasmid pJB5JI and the *R.trifolii* Sym plasmid pBR1AN, were compared in NGR234 and ANU264. This experiment was done to see if the broad-host-range cowpea strain could be changed into a *R.trifolii* or *R.leguminosarum* strain by introducing a Sym plasmid as had

been done by Brewin *et al*. (1980b) with *R.phaseoli*. This should be possible if Sym plasmids alone control all functions associated with host-range and symbiotic effectiveness. It was also possible that the NGR234 Sym plasmid only contained genes necessary for some non-specific step in the root hair curling-infection process and that genes controlling host-specific infection were elsewhere. Since Nod^- deletion mutants of *R.meliloti* could be complemented for lucerne nodulation by pJB5JI (Banfalvi *et al*., 1981), it was possible that strain ANU264 could be complemented for siratro nodulation by these exogenous Sym plasmids, if host-range controlling genes were in the chromosome or megaplasmid of NGR234.

Plasmid pJB5JI is a conjugative Sym plasmid, derived from the *R.leguminosarum* plasmid pRL1JI. This plasmid carries genes specifying nodulation and nitrogen fixation functions for symbiosis with peas. It also contains genes encoding various bacteriocinogenic properties (Beringer *et al*., 1978b). The plasmid is self transmissible at high rates between *R.leguminosarum* and *R.trifolii* strains. Selection of transconjugants carrying pJB5JI is facilitated by the presence of Tn*5* on the plasmid. When this plasmid is introduced into Nod^- derivatives of *R.trifolii* or *R.leguminosarum* strains, they behave as *R.leguminosarum* strains, giving a Nod^+Fix^+ response on peas and being Nod^- on clovers. In contrast, when pJB5JI is introduced to *R.meliloti* strains, either Nod^- deletion

mutants or wild-type strains, there is no acquisition of the ability to nodulate peas. The deletion mutants of Banfalvi *et al.*(1981) which can be complemented by pJB5JI for the nodulation of their normal host, lucerne, still do not nodulate peas.

The plasmid pBR1AN is a recombinant plasmid formed *in vivo* by some kind of recombinational exchange between pJB5JI and the endogenous Sym plasmid of the *R.trifolii* strain T1 (Djordjevic *et al.*, 1983). This plasmid retains the transmissibility of pJB5JI, the Tn*5* copy and the bacteriocinogenic properties, but now contains genes for specific symbiosis with clover rather than peas. Derivatives of *R.trifolii* and *R.leguminosarum* containing this plasmid are capable of effectively nodulating clovers. When transferred to *R.meliloti* strains, pBR1AN behaves in a similar manner to pJB5JI, that is, there is no inheritance of nodulation specificity for clovers but certain *R.meliloti* deletion mutants can be complemented to nodulate lucerne.

Plasmids pJB5JI and pBR1AN were conjugated into strains NGR234 and ANU264 from the *R.leguminosarum* donor strains 6015 (pJB5JI) and 6015 (pBR1AN), using the filter method. Transconjugants were selected on TM medium with Km at 200 *u*g ml^{-1}. The transfer rate was 10^{-2}. Ten single colonies were picked and purified on selective media and shown to be free of donor cells by streaking on TY medium where distinctive colony morphology differences between

6015 and NGR234 strains are apparent. The plasmid stability was tested by subculture on non-selective media and all strains maintained Km^R through at least four subcultures. The two Sym plasmids could be re-transferred to the R.leguminosarum strain 6015 where they behaved normally. Results of nodulation tests are summarised in Table 4.1.

Both NGR234 (pBR1AN) and NGR234 (pJB5JI) were able to nodulate siratro with no delay, forming a normal nodule number. Strains ANU264 (pJB5JI) and ANU264 (pBR1AN) were Nod^- on siratro, demonstrating that these two plasmids cannot complement ANU264 for siratro nodulation. Therefore nodulation host-range is determined by the NGR234 Sym plasmid and not by the chromosome or megaplasmid of NGR234.

On white clover, strain NGR234 was able to distort root hairs and cause stunted development of roots, especially laterals, which become short, swollen and club-like. Although no true nodules develop, a series of symmetrical swellings can occur along the root giving the appearance of beads. On peas, NGR234 caused a slight brown pigmentation in the roots but had no other effect. On subterranean clover no obvious root effects occurred.

Strain NGR234 (pJB5JI) formed abundant small nodules on peas, about 400 per plant. Surprisingly, this strain induced small nodules on white clover (Fig. 4.5), as well as causing root beading. The strain did not have an

obvious effect on subterranean clover. This is interesting since pJB5JI codes for the nodulation of peas and subterranean clover but not white clover (Djordjevic et al., 1983). ANU264 (pJB5JI) produced about 10 small Fix^- nodules per plant on peas and had a dramatic effect on the pea root causing swelling, a splitting of the epidermis and a spongy appearance. No nodules occurred on either white or subterranean clover although slight root distortion occurred on white clover.

Strain NGR234 (pBR1AN) was Nod^- on peas, but formed small white, ineffective nodules on both clover species (Fig. 4.6.a). The nodules on white clover were similar to those induced by strain NGR234 (pJB5JI). Strain ANU264 (pBR1AN) could nodulate white and subterranean clovers rapidly, forming normal-looking nodules with anthracyanin pigmentation after 10 days (Fig. 4.6.b). At this stage the nodules were the same as nodules induced by a wild-type R.trifolii strain (Fig. 4.6.c). Subsequently the ANU264 (pBR1AN) nodules did not develop and were Fix^-. It was obvious that the ANU264 (pBR1AN) nodules had progressed further than the NGR234 (pBR1AN) nodules.

Root hair effects were observed using white clover. Strain NGR234 was able to cause morphological distortions of root hairs, but not the "shepherd's crook" type of root hair curling indicative of infection. Strain NGR234 (pJB5JI) produced distortions, branching and marked root hair curling. The same kind of distortions and curling

were produced by NGR234 (pBR1AN). Strain ANU264 did not cause distortions or curling. Strain ANU264 (pBR1AN) caused extreme morphological distortions and curling whilst strain ANU264 (pJB5JI) produced a low degree of distortion or curling.

Microscopic examination of pea and clover root nodules induced by strain NGR234 (pJB5JI) showed that the nodules were devoid of bacteria, the nodule consisting of vacuolate plant cells. Very few infection threads were seen. Nodules on white clover induced by NGR234 (pBR1AN) were similar. In contrast, nodules induced by ANU264 (pBR1AN) on white clover had a normal organisation for an early nodule, with many branched infection threads and plant cells packed with bacteria. Why these bacteria did not differentiate into nitrogen fixing bacteroids is not known. Vincent (1980) described this phenotype as Bad^- (or lack of bacteroid development). The other strains can be considered Bar^- (or lack of bacterial release). The effect on peas caused by ANU264 (pJB5JI) was not examined.

Plant tests were established to see if the effectiveness of the symbiosis between siratro and NGR234 was altered by the introduction of pJB5JI and pBR1AN. An extra test was included using the plasmid pRD1, which is a R-prime plasmid derived from RP4 containing the entire *Klebsiella pneumoniae nif* gene cluster (Dixon *et al.*, 1976). As a control, strain NGR234 (RP4) was included. Both strains had been passaged through siratro nodules

with no loss of plasmids, judged by the resistance markers and gel electrophoresis. The other test strains were a pure clone of NGR234 (pJB5JI) and two derivatives of this strain which had been passaged through siratro nodules, two strains of NGR234 (pBR1AN) which had been through siratro, and the $Nod^{+}Fix^{+}$ strains NGR234 and ANU240.

Each test used twelve siratro plants grown in test tubes with the tops of the tubes exposed to sunlight in a green house. Plants were grown for twelve weeks before being harvested. The results are shown in Table 4.2.

The strains NGR234 (pBR1AN) and NGR234 (pJB5JI) were not effective in nitrogen fixation even though an adequate number of nodules had formed. Only one plant out of the total of thirty inoculated with either of these strains showed even a slight degree of effectiveness. The strain NGR234 (pRD1) was also Fix^{-} on siratro although the control strain NGR234 (RP4) was perfectly effective. The number of nodules induced by the various strains was not significantly different.

When pJB5JI was transferred to the slow-growing strain CP283 the transfer rate was extremely low. Transconjugants containing pJB5JI were all totally ineffective on siratro, and in contrast to NGR234 (pJB5JI), the slow-growing transconjugants were unable to nodulate peas (G. Bender, personal communication).

4.6 THE PLASMIDS OF NGR234: FACT AND ARTIFACT

At the start of this research programme the Eckhardt technique (1978) and the technique of Kado and Liu (1981) were the most popular methods for analysing the plasmid content of Gram-negative bacteria. Of these techniques the Eckhardt was most popular with Rhizobium since it could be performed on cells taken directly from TY plates, and the general procedure was not as tedious as the Kado method.

The Eckhardt technique (and adaptations of this method for horizontal slab gels) could only occasionally resolve plasmids of the size of the R.meliloti megaplasmid, which is as much as 450 megadaltons. So, at the start of this work it was believed that strain NGR234 contained only one plasmid of about 200 Mdal. This size was estimated from gels on strain NGR234 (pJB5JI) since pJB5JI is known to be 130 Mdal and the NGR234 plasmid banded at a position slightly above this. The size of the Sym plasmid of NGR234 was later revised by Plazinski et al. (1983) to be 310 Mdal, a figure in agreement with that of Dr. W. Broughton (Max Planck Institute, Kőln, personal communication) for another line of strain NGR234.

Infrequently it was observed that a plasmid band occurred near the origin of the gel and this hinted at the existence of a megaplasmid in NGR234. It was even thought that this band represented the entire supercoiled

chromosome entering the gel. A modification of the Eckhardt technique by Pűhler (unpublished) became available towards the end of the research of this thesis. This technique verified the existence of the megaplasmid in NGR234. It also showed that the plasmid is slightly larger than the R.meliloti megaplasmid pRme41b (see Fig. 4.1).

The NGR234 Sym plasmid was previously designated pNM3AN (Morrison et al., 1983) however this designation is not consistent with the now widely used system of Casse et al. (1979). Since rhizobia of uncertain taxonomic affiliation, such as NGR234, are usually known as Rhizobium sp., it is proposed to call the NGR234 Sym plasmid pRspNGR234a and the megaplasmid pRspNGR234b. The size of the megaplasmid pRspNGR234b is difficult to estimate, since there are no well characterised plasmids of a similar size. All that can be said is that it is similar in size to the R.meliloti megaplasmid pRme41b.

The existence of two small "plasmids" in NGR234 must be accepted as being a puzzling artifact of a modification of the Eckhardt technique. These two "plasmids" of about 20 and 25 Mdal could not be purified on caesium chloride-ethidium bromide gradients of cleared lysates of NGR234 and cannot be demonstrated using other techniques. This phenomenon is shown by Morrison et al. (1983).

4.9 DISCUSSION

The results in this chapter show that the plasmid pRspNGR234a is required for the nodulation of a range of legumes and Parasponia andersonii. The loss of the plasmid results in a block of the symbiotic interaction at the very early stages of root hair curling and infection thread formation. The cured strain ANU264 was able to adhere to root hairs and form microcolonies on the root hair surface. It was not determined if any of these root hair interactions were host-specific. Zurkowski (1981) demonstrated that root hair adhesion was a characteristic encoded on a R.trifolii Sym plasmid, pWZ2. Work on other Sym plasmid-cured R.trifolii strains (J. Badenoch-Jones, personal communication) has shown that there is no significant change in the ability to adhere to root hairs between wild-type and heat-cured strains.

The pattern of hybridisation to the nifH and nifD probes shows that at least these genes involved in nitrogen fixation are also on the plasmid. The fact that two intense hybridisation bands appeared in Southern analysis even with a number of restriction enzymes (J. Watson, personal communication) suggest that the nifH and nifD genes were not linked on the same DNA fragment, as had been found for the slow-growing cowpea Rhizobium strain CP283 (Scott et al., 1983a). However, it was conclusively proven that there are actually two separate

loci containing linked *nifH*, *nifD* and *nifK* genes on pRspNGR234*a* (Badenoch-Jones *et al.*,1984).

The aim of the mobilisation experiment using RP1::Tn*501* was to show that strain ANU265 had not suffered some unknown mutation (which prevented nodulation) with a coincidental loss of a plasmid. The fact that pRspNGR234*a* was present in the nodulating transconjugants proves its involvement in nodulation. This experiment also showed that the heat-cured strain was unaltered in other genes essential for an effective symbiosis.

The studies on the expression of the Sym plasmids pJB5JI and pBR1AN in NGR234 and ANU264 have some intriguing implications. The experiment shows that the control of host-range is carried on pRspNGR234*a*, since neither exogenous Sym plasmid could complement the heat-cured strain for nodulation of siratro. In the wild-type strain, NGR234, there was a subtle change in the behaviour of pJB5JI, with an apparent extension of infection host-range to white clover. Presumably this is the result of some interaction between the nodulation functions of these two plasmids. Microscopic examination of the pea and clover nodules showed that nodule development is blocked at the bacterial release and bacteroid development stages, depending upon which transconjugant is considered. One can conclude that these Sym plasmids do not have all the genes required for effective symbiosis with peas and clovers. The genetic

background (chromosome and megaplasmid) of NGR234 must not have the correct allelic forms of certain as yet unknown genes, which are required for effective nodulation of peas and clover. Presumably if all these genes were isolated and introduced into NGR234 it may be possible to transform NGR234 into an R.leguminosarum or R.trifolii strain. These data suggest the existence of non-Sym plasmid encoded genes which control host-range at the stage of bacterial release from infection threads or bacteroid development. Similar experiments in Chapter Five also suggest the existence of such genes.

Apart from these effects on nodulation, both pBR1AN and pJB5JI prevented expression of the effective symbiosis which normally occurs between NGR234 and siratro. The fact that pRD1 had the same effect suggests that the nif gene cluster of these plasmids is responsible since this is the only known genetic function in common between pRD1, and the two Sym plasmids. Exactly why this inhibition should occur is not known, however it may be possible to narrow the effect down to a particular gene since Downie et al. (1983b) have recently described Tn5-induced Fix^- mutations in pRL1JI (the Sym plasmid from which pJB5JI was derived). Some of these mutant plasmids may not express this inhibition of effectiveness in NGR234.

SUMMARY

1. The Sym plasmid pRspNGR234a which is the smaller of two large plasmids in NGR234 is absolutely essential for nodulation on various legumes and the non-legume, Parasponia.

2. The Sym plasmid controls the nodulation process at the root hair curling stage, one of the earliest steps in the plant-bacterium interaction.

3. Genes for nitrogenase (nifH and nifD) are carried on the Sym plasmid. These genes are duplicated in two clusters. So the Sym plasmid also determines the expression of nitrogen fixation.

4. The control of symbiotic host-range is also determined by the Sym plasmid.

Acknowledgement

Some of the results shown in this chapter have appeared in the paper "Heat-curing of a Sym plasmid in a fast-growing Rhizobium sp. that is able to nodulate legumes and the non-legume Parasponia sp." by N.A. Morrison, C.-Y. Hau, M.J. Trinick, J. Shine and B.G. Rolfe in Journal of Bacteriology, Volume 153: 527-531 (1983).

Table 4.1 Nodulation phenotypes of various transconjugant strains.

STRAIN	PLANT HOST			
	White clover	Sub-clover	Pea	Siratro
NGR234	Root distortions	Nod^-	Root browning	Nod^+Fix^+
NGR234(pJB5JI)	Nod^+	Nod^-	Nod^+	Nod^+Fix^-
NGR234(pBR1AN)	Nod^+	Nod^+	Nod^-	Nod^+Fix^-
ANU264	Nod^-	Nod^-	Nod^-	Nod^-
ANU264(pJB5JI)	Nod^-	Nod^-	Nod^+	Nod^-
ANU264(pBR1AN)	Nod^+	Nod^+	Nod^-	Nod^-

All nodules were small, blister-like and ineffective, except on siratro.

Table 4.2. Abolition of effective symbiotic ability of strain NGR234 by the introduction of foreign Sym plasmids.

STRAIN	EFFECTIVENESS
NGR234	E
ANU240	E
ANU239	E
NGR234 (pBR1AN)-1	I
NGR234 (pBR1AN)-2	I
NGR234 (pJB5JI)-1	I
NGR234 (pJB5JI)-2	I
NGR234 (pJB5JI)-3	I
NGR234 (pRD1)	I
NGR234 (RP4)	E

This experiment was performed in conjunction with Dr. M.J. Trinick, C.S.I.R.O. Division of Land Resources Management, Wembly, Perth. Nodule number was not significantly variable. Effectiveness was based on a dry weight comparison of twelve week old plants grown in tubes under greenhouse conditions. E means effective symbiosis. I means ineffective symbiosis.

Figure 4.1. Eckhardt gel analysis of plasmids in strain NGR234 and the heat-cured strain ANU264. The megaplasmid of NGR234 is clearly shown in this gel. Track 1 is NGR234, showing (a) pRspNGR234a and (b) pRspNGR234b. Track 2 is the heat-cured strain ANU264. It is obvious that the heat-cured strain has lost the smaller Sym plasmid (pRspNGR234a) but retains the megaplasmid (pRspNGR234b). Track 3 is the R.meliloti strain 41 which has (a) the cryptic 140 Mdal plasmid pRme41a and (b) the megaplasmid pRme41b, which is thought to be 450 Mdal (A. Kondorosi, personal communication). This gel was done by Dr. J. Plazinski.

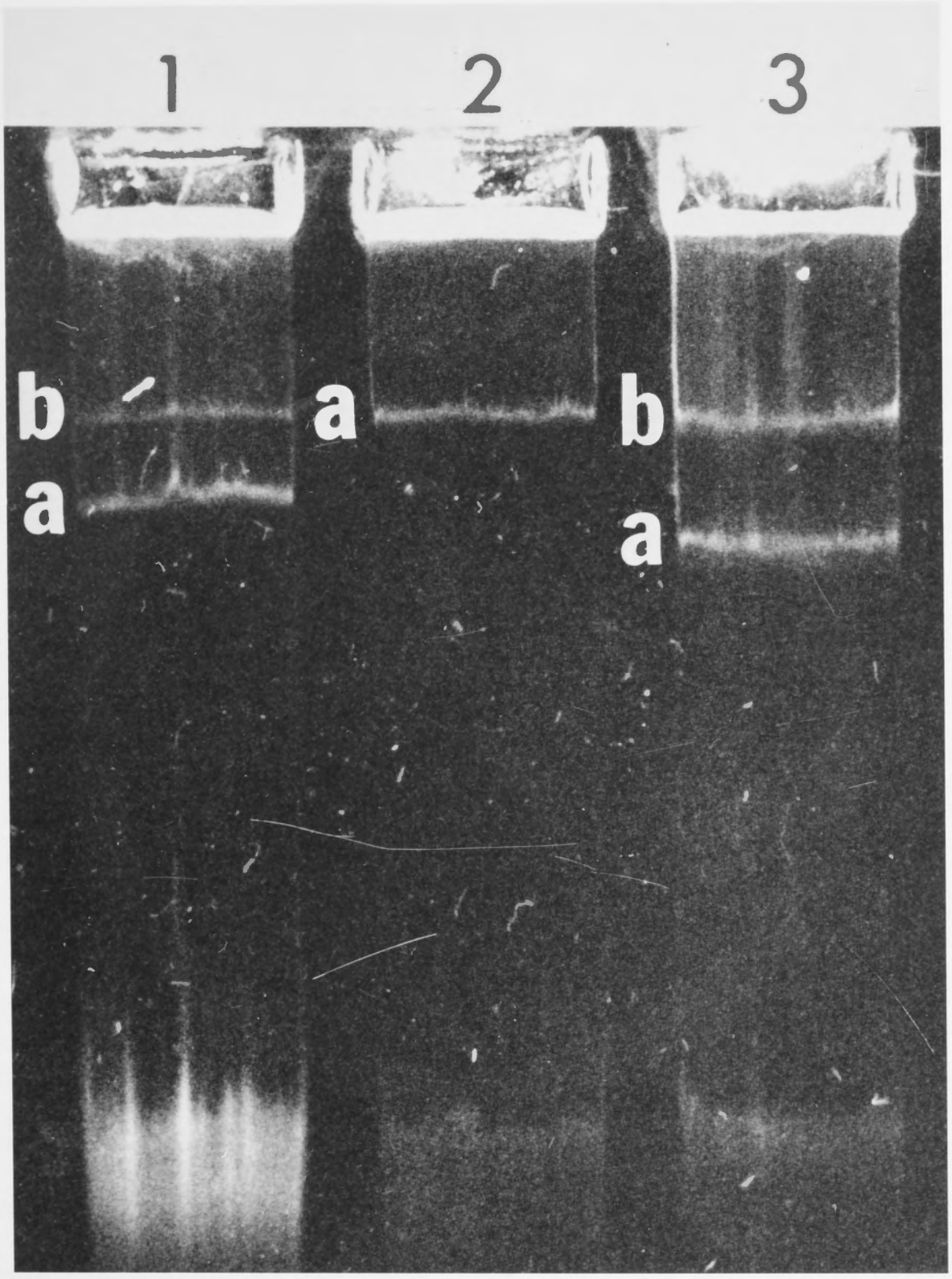
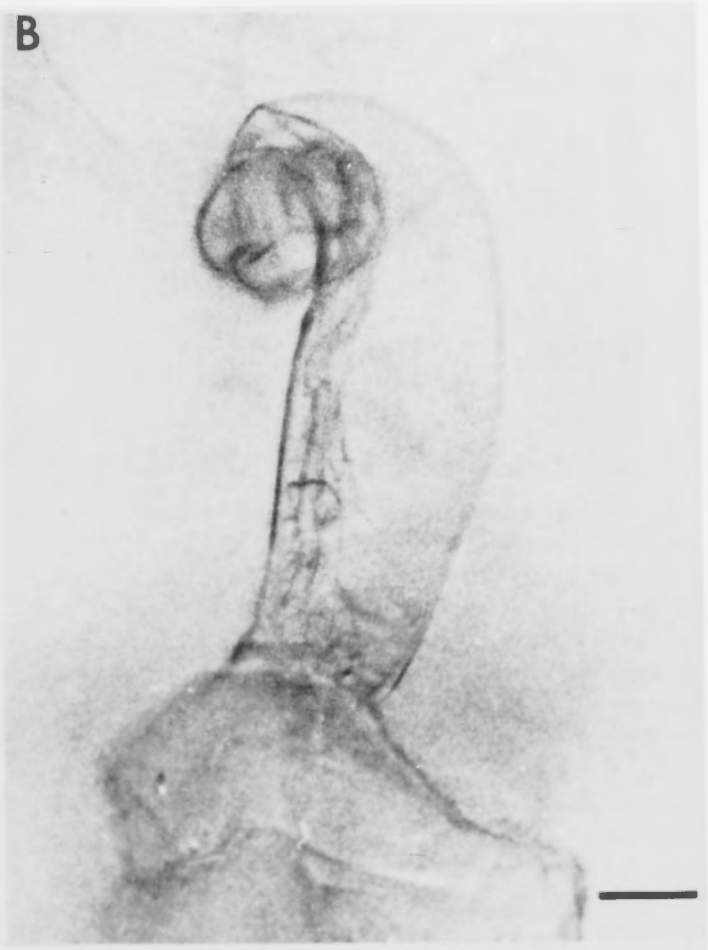

1
2
3
b
a
a
b
a

Figure 4.2. Loss of the early nodulation function of root hair curling in strain ANU264. A. Normal extreme root hair curling and distortions induced on siratro by strain ANU240. B. An infection thread seen in a root hair above a small nodule; strain ANU240. C. Siratro root hairs inoculated with ANU264, showing no morphological aberrations or root hair curling.


A


B

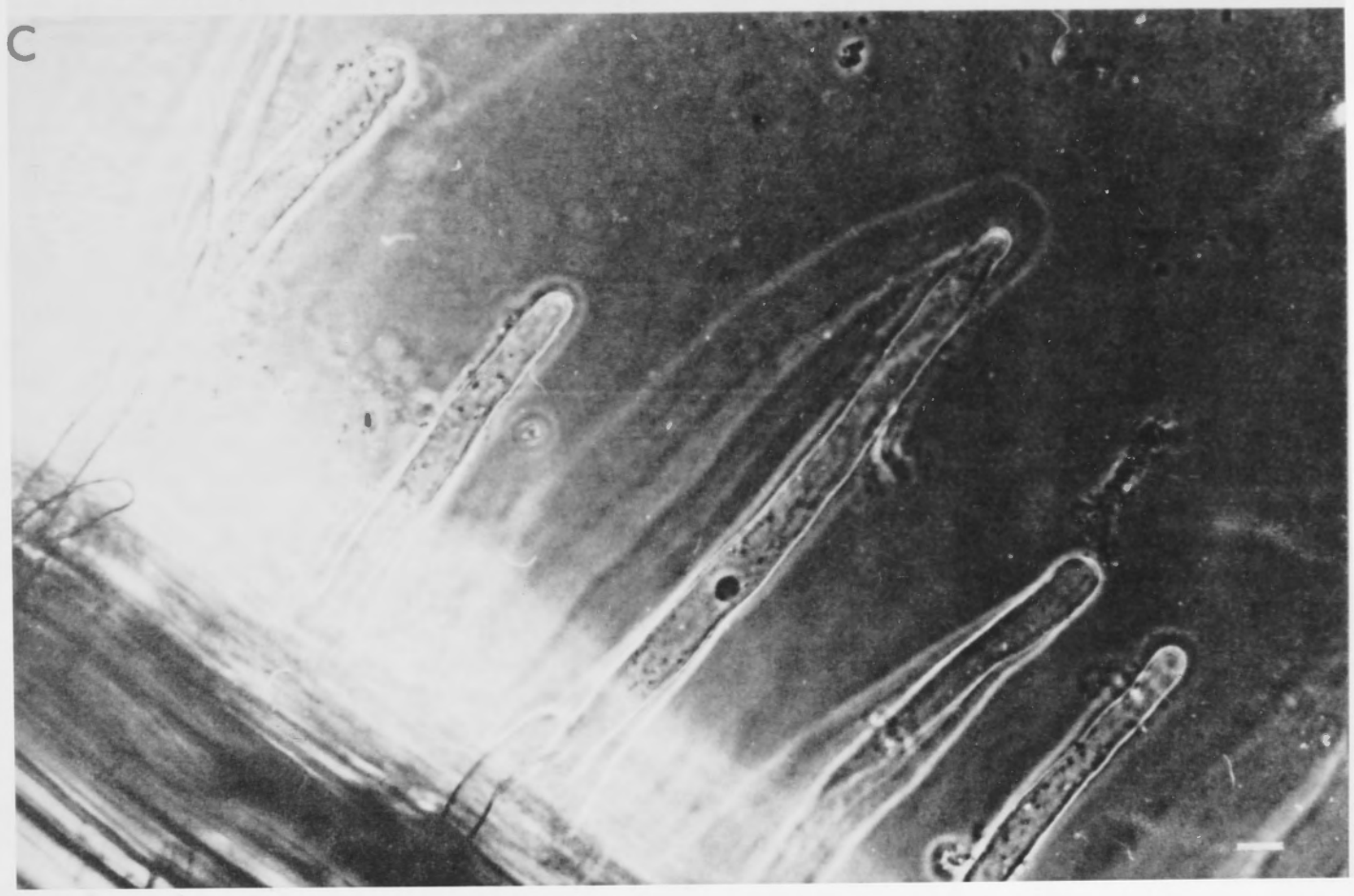
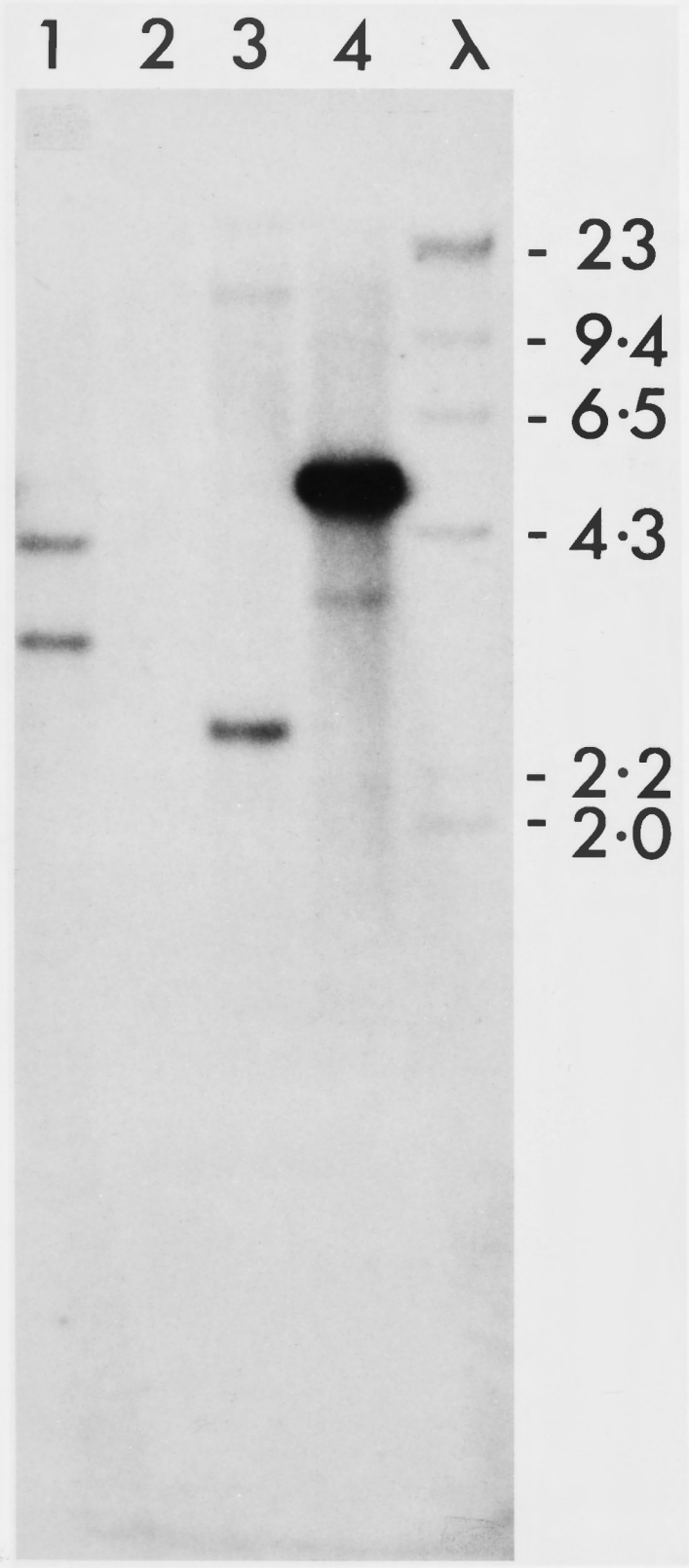

C

Figure 4.3. Identification of the nifH and nifD genes in strain NGR234. Total DNA of various strains was cut with EcoRI, resolved in 1% agarose gels, blotted and probed with pRtnif-2, a recombinant plasmid containing the R.trifolii SU329 nifH gene and a portion of the nifD gene. Strains are: track 1, ANU240; track 2, ANU264; track 3, ANU264 (pJB5JI); track 4, ANU264 (pBR1AN). Molecular weight markers are end-labelled HindIII cut lambda DNA.


1
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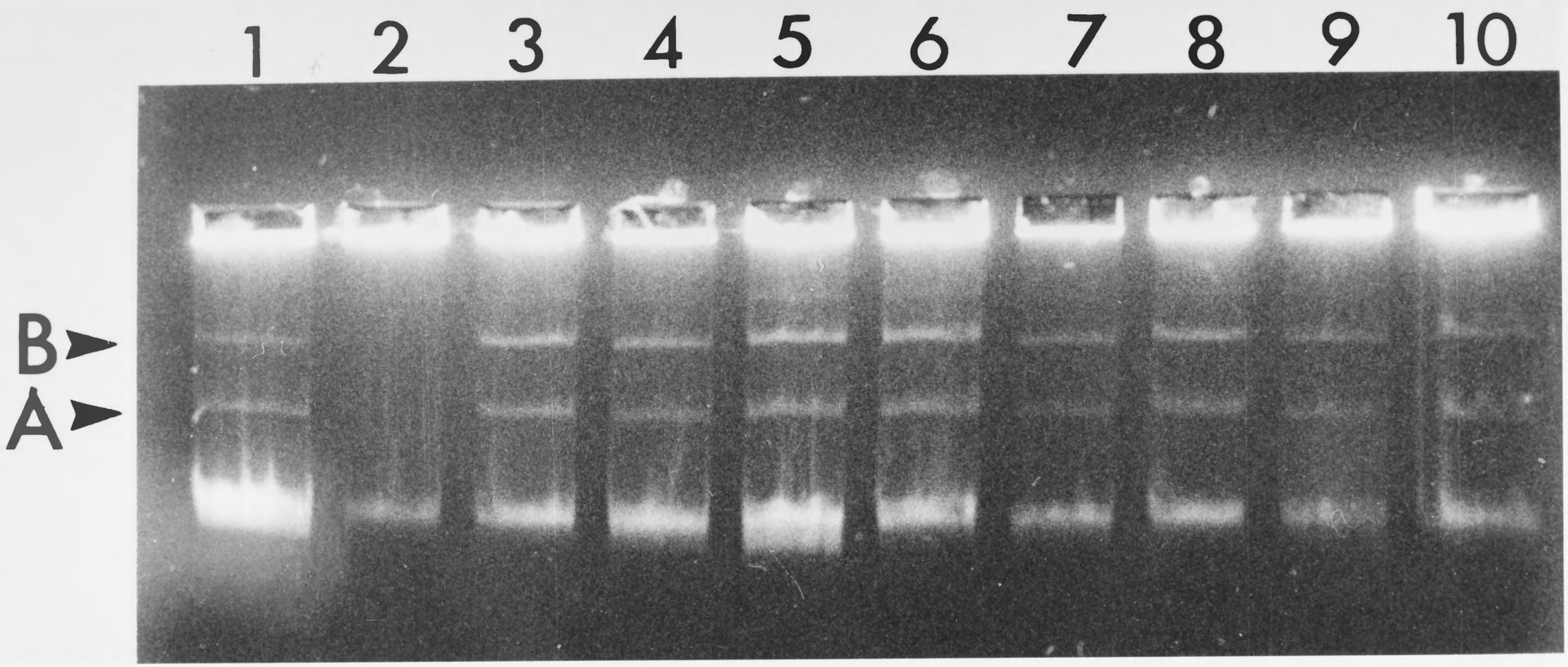
3
4
λ
- 23
- 9·4
- 6·5
- 4·3
- 2·2
- 2·0

Figure 4.4. Agarose gel electrophoresis to show mobilisation of pRspNGR234a. Track 1 is the donor strain ANU239 (RP1::Tn501). Track 2 is strain ANU265. Tracks 3 to 10 are separate nodulating transconjugants which are Rif^S, Sm^R, Sp^R, Km^R, Tc^R and Hg^R. Most tracks have not resolved the megaplasmid pRspNGR234b. Tracks 5 and 6 show faint megaplasmid bands. A. represents the plasmid RP1::Tn501. B. represents pRspNGR234a.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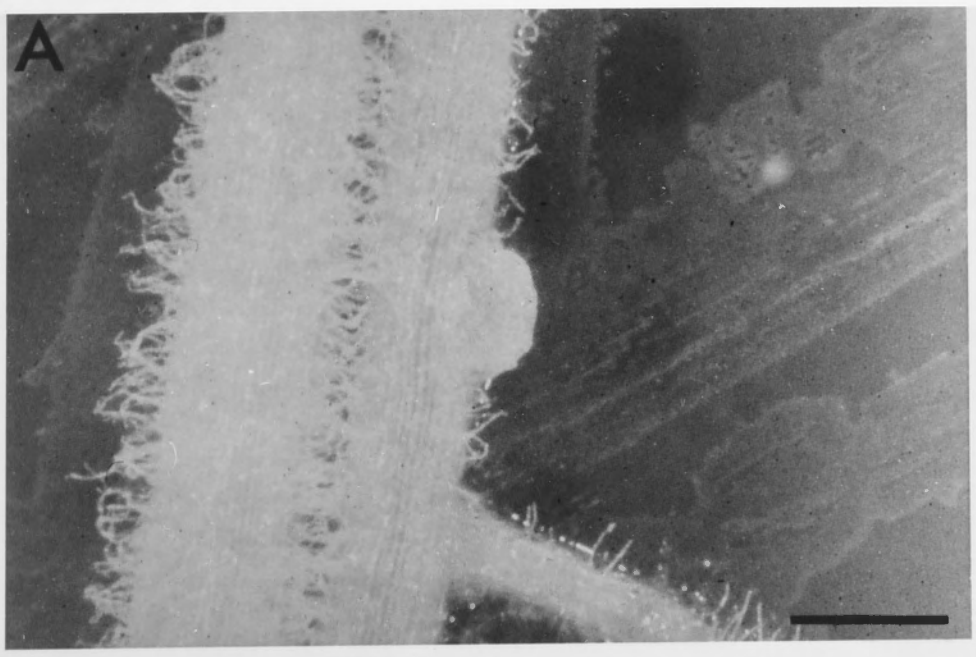
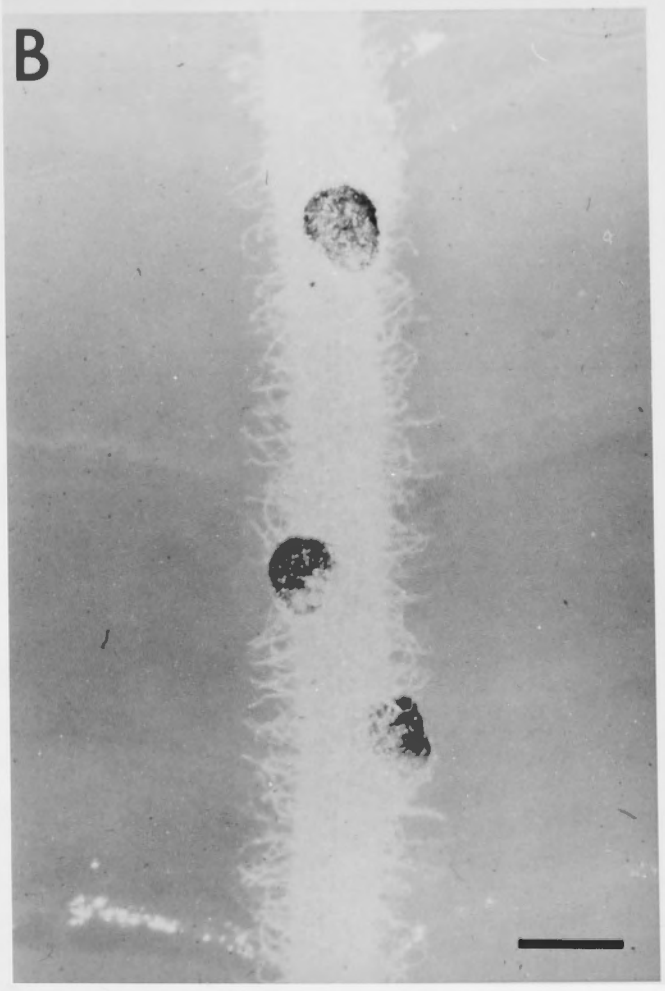
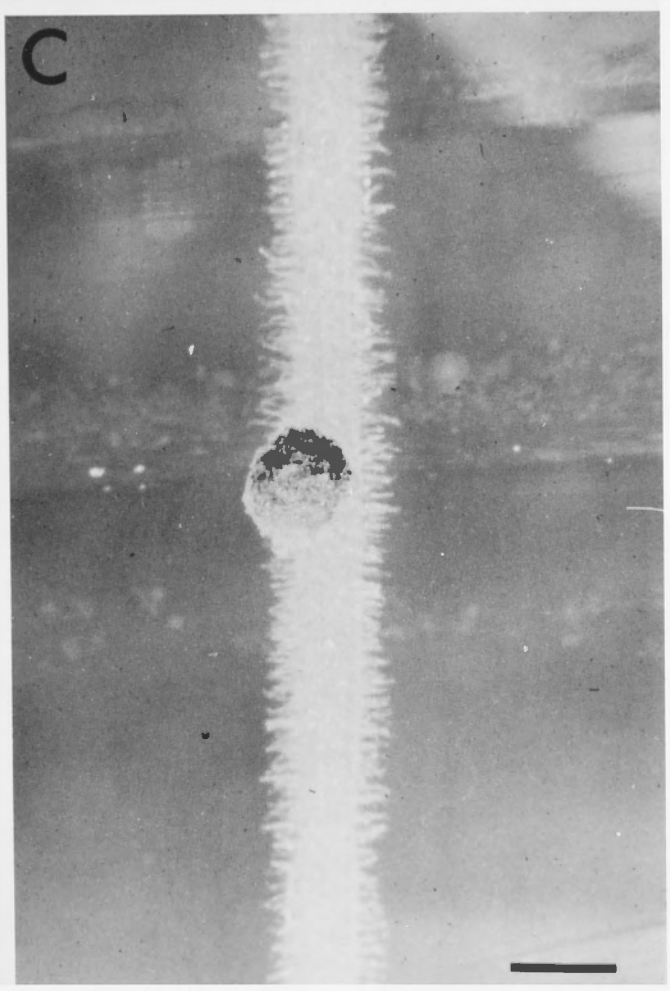
6
7
8
9
10
B
A

Figure 4.5. Micronodules induced by strain NGR234 (pJB5JI) on white clover.

Figure 4.6. Nodules induced on white clovers by transconjugant strains: A. NGR234 (pBR1AN); B. ANU264 (pBR1AN); C. Wild-type R.trifolii strain. All nodules are 10 days old. Bar represents 1 mm.


A


B


C

CHAPTER FIVE

MOBILISATION OF THE NGR234 SYM PLASMID TO OTHER STRAINS OF *RHIZOBIUM* AND *AGROBACTERIUM*

5.1 INTRODUCTION

It has been widely shown that large Sym plasmids encode genes for nodulation and nitrogen fixation in the fast-growing temperate rhizobia, *R.trifolii*, *R.leguminosarum*, *R.meliloti* and *R.phaseoli*. To date such Sym plasmids have not been reported in slow-growing rhizobia of any type. Fast-growing *R.japonicum* strains (Chapter Three; Appelbaum *et al.*, 1984) and at least one other fast-growing cowpea strain, IHP100, have Sym plasmids (J. Plazinski, personal communication).

Self-transmissible Sym plasmids have been found in *R.leguminosarum* (Brewin *et al.*, 1980a) and *R.trifolii* (Hooykaas *et al.*, 1981). A recombination between the *R.leguminosarum* Sym plasmid pJB5JI and an endogenous *R.trifolii* Sym plasmid gave rise to the self-transmissible plasmid pBR1AN, which carries genes for clover nodulation and nitrogen fixation (Rolfe *et al.*, 1981a). Scott and Ronson (1982) have used a cointegrate between the *Inc*P1 plasmid R68.45 and a *R.trifolii* Sym plasmid to transfer

symbiotic genes to a large number of bacteria. Recently, a R.meliloti megaplasmid was mobilised to other bacteria (Kondorosi et al., 1982). These plasmids have allowed a comparison of the behaviour of various symbiotic genes in different Rhizobium species.

Chapter Four showed that NGR234 has a large Sym plasmid which can be lost through a process of heat-curing and can be mobilised at a low frequency by RP1::Tn501. In this chapter an experimental plan was devised to construct a Sym plasmid which was capable of being mobilised at a high frequency to other bacteria. This plasmid was used to investigate the expression of "cowpea" symbiotic genes in other Rhizobium strains. One of the main questions to be answered was whether the NGR234 Sym plasmid would be more "successful" in fast-growing rhizobia (since NGR234 is fast-growing) or in slow-growing rhizobia (the normal endosymbionts of the plants in the NGR234 host range).

5.2 CONSTRUCTION OF A MOBILISABLE COINTEGRATE SYM PLASMID

When pSUP1011 was used for transposon mutagenesis in strain ANU240, about 20% of putative Tn5-containing derivatives had also inherited Cm^R, the other marker on pSUP1011 (Chapter Six). These strains are suspected to have arisen by cointegrative "rescue" of pSUP1011, since Tn5 is known to mediate cointegration events (Hirschel et al., 1982). As well as rescuing Km^R and Cm^R, integration

would result in the insertion of the mobilisation site (Mob), which is carried on pSUP1011. If such cointegrations were in the chromosome it could provide a site for mobilising the chromosome at high frequency. If the cointegration was in the Sym plasmid then this could create a mobilisable Sym plasmid-pSUP1011 cointegrate.

The plasmid pSUP1011 was conjugated from the E.coli strain SM10-1011 to the Rhizobium strain ANU269 (a Rif^R, Sm^S, Sp^S, NGR234 derivative), which carries the IncP1 plasmid pJB3JI. This plasmid was used to provide the necessary transfer functions for the mobilisation of possible cointegrates. Cm^R was used to select possible cointegrates rather than bona fide Tn5 transposition derivatives. Rif was used to select against the E.coli donor. The rate of transfer of Cm^R was 5×10^{-7}.

It was thought that a cointegration between pSUP1011 and the NGR234 Sym plasmid would be a fairly common event due to the large size of the Sym plasmid. Three hundred Cm^R,Km^R ANU269 clones were purified and separately mated with strain ANU265 on TY medium. Strain ANU265 is a Rif^S, Sm^R, Sp^R, heat-cured Nod^- NGR234 derivative, which was used previously (Chapter Four) as a recipient for the mobilisation of the NGR234 Sym plasmid. Nearly all of the Cm^R,Km^R ANU269 clones transferred Km^R at a detectable rate to ANU265. The ANU265 transconjugants were purified to single colonies and individually inoculated onto siratro. None formed nodules, indicating that the cointegration

between pSUP1011 and the Sym plasmid is not a common event.

Since the cointegration must be a rarer event than previously thought, a larger number of possible cointegrates had to be tested. Over 3000 Cm^R ANU269 transconjugants were subcultured three times on selective media by replica plating. No contamination by the E.coli donor could be detected after this purification method. The purified Cm^R ANU269 clones were replica plated onto TY medium, incubated overnight, and then replica plated onto 16-hour old lawns of strain ANU265 on TY medium. Conjugation was allowed to proceed overnight before the transfer of Km^R and Cm^R into ANU265 was selected by replica plating onto appropriate selective media. A very high rate of transfer of both Cm^R and Km^R was found. Subsequent purification of ANU265 colonies was done using Km^R due to the poor expression of Cm^R in the presence of Sp. Transconjugant ANU265 colonies were purified by replica plating through three selective subcultures. No contamination by ANU269 cells could be detected by replica plating on TY Rif medium. Purified Km^R ANU265 colonies were washed off selective plates en masse and diluted by 10^0, 10^{-1} and 10^{-2}. These dilutions were used to inoculate ten siratro plants each. After two weeks, fourteen nodules had developed on only two plants inoculated with the undiluted suspension. Bacteria were reisolated from these nodules and tested for various

characteristics. All had the distinctive colony morphology of strain NGR234 derivatives and their Nod^+ ability was stable in subculture. All strains were Sm^R and Sp^R (characteristic of ANU265), Tc^R (carried on pJB3JI), Km^R and Cm^R (carried on pSUP1011). All strains were sensitive to Rif. When plasmid profiles were checked, all had the 38 Mdal plasmid pJB3JI, and a band corresponding in size to the NGR234 Sym plasmid. Hybridisation using a Tn*5* probe showed that the Sym plasmid carried a copy of Tn*5*. One of these strains was chosen for further work. This strain was designated ANU271 and the cointegrate Sym plasmid was designated pNM4AN.

5.3 TRANSFER OF SIRATRO NODULATION ABILITY TO NOD^- MUTANTS OF *R.MELILOTI*, *R.LEGUMINOSARUM*, *R.TRIFOLII* AND *A.TUMEFACIENS*

Strain ANU271 was able to transfer Km^R at a rate of about 10^{-2} to the Nod^- *R.meliloti* strain ZB157. This strain has a deletion in the *R.meliloti* megaplasmid pRme41*b* which has removed all of the root hair curling genes (Banfalvi *et al.*, 1981), but still retained a locus implicated in host specificity (Kondorosi *et al.*,1983). All tested *R.meliloti* ZB157 transconjugants were Nod^- on lucerne, but were able to nodulate siratro, forming large partially effective nodules after two weeks. The

interiors of the nodules were pink indicating that leghaemoglobin was present, and acetylene reduction assays gave about 50% of the rate of strain ANU240 nodules. ZB157 (pNM4AN, pJB3JI) could retransfer pNM4AN to strain ANU265. The ANU265 transconjugants were $Nod^{+}Fix^{+}$ on siratro. The plasmid pNM4AN and pJB3JI could be seen to be separate entities in gel analysis of ZB157 (pNM4AN, pJB3JI) (Fig. 5.1).

Strain ANU271 could transfer pNM4AN at a frequency of about 10^{-2} to the R.leguminosarum strain 6015. This strain has a deletion encompassing the entire symbiotic gene region (Downie et al., 1983b). The R.leguminosarum strain 6015 transconjugants were Nod^{-} on peas but were able to nodulate siratro, forming large nodules on the tap root after a slightly delayed time, as well as some nodules with white epidermal outgrowths (Fig. 5.2.a). The symbiosis was not effective in nitrogen fixation as indicated by poor growth of the plants, a lack of red pigmentation in the nodules and an absence of acetylene reduction activity. A similar result was found using the plant Desmodium intortum. Strain 6015 (pNM4AN, pJB3JI) clones were able to transfer Km^{R}, Cm^{R} and Tc^{R} to ANU265. The resultant ANU265 (pNM4AN, pJB3JI) were again $Nod^{+}Fix^{+}$ on siratro, demonstrating that pNM4AN had not lost its symbiotic nitrogen fixation ability as a result of the passage through R.leguminosarum.

The plasmid pNM4AN could be transferred from ANU271,

6015 (pNM4AN, pJB3JI) and ZB157 (pNM4AN, pJB3JI) to the R.trifolii strain ANU1064 again at about 10^{-2} frequency. ANU1064 is a heat-cured derivative of ANU1002 (a Rif^R mutant of R.trifolii strain 5 from Rothamsted). R.trifolii ANU1064 (pNM4AN, pJB3JI) was able to nodulate siratro and Desmodium intortum in a similar fashion to 6015 (pNM4AN, pJB3JI), but could not nodulate clovers. ANU1064 (pNM4AN, pJB3JI) could transfer pNM4AN back to ANU265, 6015 and ZB157. The transconjugant strains produced from these matings had the same nodulation phenotypes as the corresponding strains derived from matings with ANU271. This indicates that the nodulation phenotype of a particular strain carrying pNM4AN is not dependent on the donor used.

Strains ANU271, ZB157 (pNM4AN, pJB3JI), 6015 (pNM4AN, pJB3JI) and ANU1064 (pNM4AN, pJB3JI) could transfer pNM4AN to the A.tumefaciens strain C58 and its avirulent mutant A136. Both C58 (pNM4AN, pJB3JI) and strain A136 (pNM4AN, pJB3JI) had the same nodulation phenotype. On siratro these strains formed nodules mostly on the lateral roots, with a few small nodules on the tap roots. The lateral root nodules had "furry" white epidermal outgrowths and a callused appearance (Fig.5.2.b). This type of nodulation is the same as described in Chapter Six for the nodulation-defective mutant L10 (ANU1260) and various auxotrophs. On Desmodium intortum ineffective micronodules developed on the main

tap root. This is again similar to the mutant L10.

Strains C58 (pNM4AN, pJB3JI) and A136 (pNM4AN, pJB3JI) could transfer pNM4AN back to ANU265, where a normal Nod^+Fix^+ response on siratro was regained. Some clones of A136 (pNM4AN, pJB3JI) were unable to form nodules or nodule-like growths on siratro, yet retained Km^R, Cm^R. These clones could still retransfer an apparently normal pNM4AN back to ANU265. C58 (pNM4AN, pJB3JI) strains could still induce crown gall tumours on Datura plants (R. Ridge, personal communication).

The R.leguminosarum, R.trifolii, R.meliloti and A.tumefaciens strains mentioned above, all stably maintained pNM4AN through non-selective subculture and through siratro nodules. The plasmid could be transferred between these strains at a high frequency and always gave the same symbiotic response in a particular recipient regardless of the donor. No spontaneous loss of either Km^R or Cm^R, or both together, in any strain harbouring pNM4AN was noted. The only instability found was an occasional loss of pJB3JI from 6015 (pNM4AN, pJB3JI) strains. These strains could still nodulate siratro but could not transfer Km^R or Cm^R to ANU265. Conjugation of pJB3JI from E.coli back into such strains reinstated the mobilisability of pNM4AN. Again, it was possible to co-transfer Km^R, Cm^R and Nod^+Fix^+ (siratro) phenotypes to strain ANU265. These results demonstrate that pNM4AN and pJB3JI exist as two distinct entities; and that pNM4AN

requires pJB3JI for its mobilisation. It also shows that pNM4AN is not capable of self-transmission.

5.4 HOST-RANGE NODULATION GENES ARE CARRIED ON pNM4AN

R.meliloti strain ZB157 (pNM4AN, pJB3JI) was selected for further assays of symbiotic ability on a "diagnostic" sample of the plants nodulated by NGR234. The plants were chosen because they represent major taxonomic sub-tribes. ZB157 (pNM4AN, pJB3JI) was able to nodulate Desmodium intortum (Desmodiae), Leucaena leucocephala (Mimosadeae), and Parasponia andersonii (Ulmaceae). On Leucaena leucocephala the nodules were defective and callus-like, occurring mainly at root junctions (Fig. 5.2.c and d). This phenotype is similar to that of A136 (pNM4AN, pJB3JI) on siratro. Although this difference in nodulation response is apparent, these results are sufficient to conclude that the ability to infect a range of plants is carried on the Sym plasmid.

It is worthwhile to observe at this stage that siratro and Desmodium intortum have globose determinate nodules (like soybean), and that Leucaena leucocephala has an indeterminate nodule structure (like clover, pea or lucerne) (Goodchild, 1977). The Parasponia nodule has yet another structure, with a central vascular bundle surrounded by bacteroid-containing tissue, and generally like a modified lateral root (Becking,1977).

5.5 MICROSCOPY

Microscopy was done entirely by Mr. R. Ridge and is included in this thesis by permission and is only presented for clarity.

Siratro nodules infected by a wild-type $Nod^{+}Fix^{+}$ Rhizobium strain are characteristically globose in shape with a determinate meristem, like a soybean nodule (Allen and Allen, 1981). Internally, bacteria are released from infection threads into plant cells where nitrogen fixation occurs. Usually, about 50% of the internal nodule cells of siratro become infected by the Rhizobium (Fig. 5.3.a) and the differentiation of bacteria into bacteroids is not as pronounced as with nodules of white clover, lucerne or pea. Starch granules are also characteristic of the internal nodule cells. Normal nodules usually have small amounts of white "furry" epidermal outgrowths on the exterior.

Nodules induced by strain ZB157 (pNM4AN, pJB3JI) were not remarkably different from normal nodules (Fig. 5.3.b and c). The globose nodules induced by strain 6015 (pNM4AN, pJB3JI) which were examined had a complete lack of bacterial release (Fig. 5.3.d and e).

White "furry" nodules induced by any strain had similar internal structures (Fig. 5.3.f). The cells of the cortex are swollen abnormally while the central region

appears to contain vascular tissue. The epidermal outgrowths, which appear white on the exterior of the nodule, are devoid of cytoplasm. There are no zones of bacterial infection in the entire structure and infection threads have not been observed. The mode of induction of these organs is therefore enigmatic. Nodules induced by R.trifolii transconjugants were not examined.

5.6 TRANSFER OF pNM4AN TO OTHER BACTERIA

No transfer of Km^R could be detected in matings between ANU271 and the slow-growing cowpea Rhizobium strains CP283 and CB756 or the Parasponia endosymbiont NGR231. In the same matings the transfer of pJB3JI (Tc^R) could be detected at a rate of about 10^{-3}. Since pNM4AN uses the pJB3JI transfer system it must be entering the recipient cells at a rate similar to that of pJB3JI. Subsequent events which prevent the maintenance of pNM4AN are not understood. Attempts to transfer pNM4AN to a strain of Azospirillum brasilense had much the same result except that two Km^R clones were isolated, which appeared to have arisen from Tn5 transposition from pNM4AN (J. Plazinski, personal communication).

Km^R and Cm^R could be transferred to E.coli from ANU271 at a rate of about 10^{-5}. Tc^R could be transferred at a much higher rate. Km^R,Cm^R E.coli transconjugants examined for plasmid content had pSUP1011 and pJB3JI as

independent plasmids, whilst no trace of the Sym plasmid was found. As expected, these Km^R,Cm^R,Tc^R *E.coli* strains could transfer Km^R to ANU265 at low rates (like Tn*5* mutagenesis using pSUP1011). These results suggest that the pSUP1011 can be rescued from the cointegrate pNM4AN at a low frequency by mobilisation to *E.coli*. In *E.coli* the pSUP1011 can replicate and is not lost as it would be in *Rhizobium* after excision. These results also demonstrate that the entire pNM4AN molecule is not capable of replication in *E.coli*. The replication system of pSUP1011 probably cannot cope with such a large plasmid, while the Sym plasmid replication system seems incapable of functioning in *E.coli*. Although a cointegrate between R68.45 and a *R.trifolii* Sym plasmid is capable of replication in *E.coli* (Scott and Ronson,1982), no reports exist in the literature claiming that a Sym plasmid can replicate independently in *E.coli*.

5.8 DISCUSSION

The constructed mobilisable Sym plasmid pNM4AN is stably maintained, and expresses nodulation functions in classical fast-growing *Rhizobium* and *Agrobacterium* strains. This extends the view of Trinick (1980) that the ability to nodulate cowpea group plants is not an exclusive trait of slow-growing *Rhizobium* strains. The strong expression of nodulation functions in the pNM4AN

transconjugant strains suggests that the basic process of nodulation is the same in cowpea group plants[1] as in other legumes. The nodulation genes of slow-growing cowpea *Rhizobium* strains, and presumably *R.japonicum* , probably function in a manner similar to the nodulation genes in the more studied fast-growing strains.

Since root hair curling and infection threads have been seen in cowpeas and soybeans (Dart,1977; Bhuvaneswari *et al.*,1980; Bhuvaneswari *et al.*,1981; Turgeon and Bauer, 1982) it reasonable to expect that the biochemical mechanism behind nodulation in these different legumes must be very similar to that in clovers, peas and medics. This is perhaps not surprising, since these plants are all legumes. However, the *R.meliloti* strain ZB157 (pNM4AN, pJB3JI) is also able to nodulate the non-legume, *Parasponia*. This implies that the process of nodulation in *Parasponia* is similar to that in legumes, even though root hair curling does not occur on *Parasponia*. It is sure that genes for nodulation of legumes and *Parasponia* exist on the NGR234 Sym plasmid. Further detailed analysis of these nodulation genes is required to decide whether the same genes are active in the nodulation of both legumes and *Parasponia*. Since many fast-growing *Leucaena* rhizobia and slow-growing cowpea rhizobia can nodulate *Parasponia* (Trinick and Galbraith, 1980), it is unlikely that all these strains carry cryptic *Parasponia* nodulation genes, as well as genes for legume nodulation.

[1] Referring to cowpea group plants which have the infection thread invasion mechanism, rather than the lateral root primordia invasion mechanism described by Chandler (1978) and Chandler *et al.*, (1982).

R.meliloti strain ZB157 (pNM4AN, pJB3JI) was Fix^+ on siratro. This result suggests that ZB157 and NGR234 must be very similar with regard to chromosomal genes involved in nitrogen fixation (described by Forrai et al.,1983). It is known that ZB157 retains nifH and nifD but has lost some other gene essential for nitrogen fixation. The extent of expression of the endogenous nif genes of ZB157 is not known, but at least some pNM4AN nif genes must be functioning in ZB157. It is interesting that the different biochemistry of the globose determinate nodules does not prevent nitrogen fixation by the R.meliloti organism. It should be possible to use pre-existing mutants of R.meliloti in studies of requirements for nif expression in siratro.

This implied similarity between R.meliloti and NGR234 breaks down when the work of Kondorosi et al. (1983) is considered. They constructed a mobilisable derivative of the R.meliloti strain 41 Sym plasmid by cointegrating a Mob site in the nifH gene. This plasmid (pRme41b:pAK11) could be mobilised (like pNM4AN) to other bacteria but unfortunately could not confer a Fix^+ phenotype. Wong et al. (1983) studied the expression of this plasmid in a derivative of NGR234, finding that the transconjugant could only produce tumour-like growths on lucerne. These "nodules" were devoid of bacteria and were Fix^-. NGR234 is known to be able to produce tumourous growths on lucerne (W. Broughton, personal communication)

and was reported by Trinick (1980) to be able to nodulate lucerne irregularly. Hopefully, these apparently complex biological phenomena will become easier to understand with a more detailed biochemical understanding of the process of nodulation.

So far, studies on the expression of Sym plasmids in different rhizobia (summarised in Table 5.1) has lead to the conclusion that R.trifolii, R.leguminosarum and R.phaseoli belong to the same species (Downie et al., 1983a). With these bacteria the resident Sym plasmid dictates the host range of nodulation and nitrogen fixation. That is, a R.trifolii strain can be converted to a R.leguminosarum by the introduction of a Sym plasmid. Hybrid strains with increased effective host-range are possible. In these studies R.meliloti has stood apart. Introducing R.trifolii or R.leguminosarum Sym plasmids does not permit the R.meliloti to nodulate clovers or peas. The NGR234 Sym plasmid expresses the Nod functions in all these Rhizobium species but so far only expresses the nitrogen fixation functions in R.meliloti.

The R.leguminosarum strain 6015 has lost the entire symbiotic region of about 50 kb (Downie et al., 1983b), so the reason for the Fix^- phenotype of 6015 (pNM4AN, pJB3JI) is probably not the sort of interference between Sym plasmids described in Chapter Four. The same can be said for R.trifolii strain ANU1064, which has lost the entire Sym plasmid. Many more R.leguminosarum and R.trifolii

strains would have to be tested before concluding that the lack of fixation by pNM4AN is a general feature of these species.

The microscopy of Mr. R. Ridge showed a complete lack of bacteroid material in the root nodule induced by 6015 (pNM4AN,pJB3JI). Failure of transconjugant strains to be released from infection threads into plant cells has been noted by Wong et al. (1983) and Van Brussel et al. (1982) in similar experiments. Hooykaas et al. (1981) found that a A.tumefaciens strain (essentially the same as A136) carrying an R.trifolii Sym plasmid could be released from infection threads into clover nodule cells. These results may reflect phenomena associated with the test plant.

The result with pNM4AN in the R.meliloti strain suggests that the NGR234 Sym plasmid does not have all the genes required for effective symbiosis with L.leucocephala even though it apparently has all the requirements for effective nodulation of siratro. Either that, or the "genetic background" of ZB157 is not appropriate for an effective symbiosis with L.leucocephala. NGR234 must have genes which promote a normal symbiosis with siratro, L.leucocephala, and the other plants in the NGR234 host range. In Chapter Three it was shown that similar types of bacteria had puzzling differences in effective host range. Chapter Four showed that the "genetic background" of NGR234 (the heat-cured strain, ANU264) was not appropriate

for symbiotic nitrogen fixation with peas or clovers, since ANU264 transconjugant strains carrying pJB5JI and pBR1AN were unable to give an effective response on peas or clovers, respectively. The result with pNM4AN in R.trifolii and R.leguminosarum on siratro, and with R.meliloti (on L.leucocephala) may be another example of the same phenomenon.

The plasmid pNM4AN could not be transferred to cowpea or Parasponia type rhizobia. It is not known whether Sym plasmid replication functions are encoded on the plasmids themselves or are supplied in trans by some other replicon. Fast-growing Rhizobium species and A.tumefaciens used in this study must have the systems for Sym plasmid maintenance. The slow-growing rhizobia which do not have Sym plasmids seem to be incapable of maintaining pNM4AN. So it seems that the NGR234 Sym plasmid has little affinity for the slow-growing cowpea rhizobia (which have the same plant hosts as NGR234). The answer to whether the Nod genes on this plasmid are derived from a slow-growing strain or are independently evolved from the Nod genes of a fast-growing strain from another cross-inoculation group (such as the Leucaena rhizobia) must await further study.

SUMMARY

1. A suicide plasmid which contains the ori-T (Mob) region of RP4 was cointegrated *in vivo* with pRspNGR234*a*, the NGR234 Sym plasmid, to create pNM4AN. This plasmid could be mobilised to other bacterial strains.

2. *R.meliloti*, *R.trifolii*, *R.leguminosarum* and *A.tumefaciens* could all infect siratro, with varying success, when carrying pNM4AN.

3. *R.meliloti* carrying pNM4AN was partially effective in symbiosis with siratro.

4. A host range phenomenon occurred after infection when *R.meliloti* strain ZB157 (pNM4AN, pJB3JI) was inoculated on *Leucaena*. This was manifested by an inability of the transconjugant strain to elicit normal nodule development on *Leucaena*.

5. The NGR234 Sym plasmid was stable in fast-growing strains but could not be transferred to slow-growing strains.

Table 5.1 Cum ulative data concerning the expression of foreign Sym plasmids in nodulation-deficient *Rhizobium* and *Agrobacterium*.

		STAGE OF NODULATION REACHED										
		Nodulation of new host					Nodulation of normal host					
Strain	Plasmid	Hac	Inf	Nod	Bar	Fix	Hac	Inf	Nod	Bar	Fix	Source
A.tumefaciens												
A136	pJB5JI	+	+	+	-	-	Not applicable					2
	pBR1AN	+	+	+	-	-						3
	pRt5*a*	+	+	+	+	-						1
	pRme41*b*:pAK11	+	+	D	-	-						4
	pRmSL26	+	+	+	-	-						6
	pNM4AN	+	+	D	-	-						5
R.leguminosarum												
6015	pJB5JI	----------					+	+	+	+	+	7
	pBR1AN	+	+	+	+	+	-	-	-	-	-	3
	pRme41*b*:pAK11	N.T.					N.T.					
	pRmSL26	+	-	-	-	-	-	-	-	-	-	3
	pNM4AN	+	+	+	-	-	-	-	-	-	-	5

Table 5.1 Continued.

Strain	Plasmid	Nodulation of new host Hac	Inf	Nod	Bar	Fix	Nodulation of normal host Hac	Inf	Nod	Bar	Fix	Source
R.trifolii												
ANU1064	pJB5JI	+	+	+	+	+	-	-	-	-	-	3
	pBR1AN	----------					+	+	+	+	+	3
	pRme41b:pAK11	N.T.										
	pRmSL26	+	-	-	-	-	-	-	-	-	-	3
	pNM4AN	+	+	+	-	-	-	-	-	-	-	5
R.meliloti												
ZB157	pJB5JI	+	-	-	-	-	+	+	+	+	+	3,4
	pBR1AN	+	-	-	-	-	+	+	+	+	+	3
	pRme41b:pAK11	N.T.										
	pRmSL26	----------					+	+	+	+	+	3,4,10
	pNM4AN	+	+	+	+	+	-	-	-	-	-	5
R.meliloti												
ANU1004	pJB5JI	+	-	-	-	-	-	-	-	-	-	3,4,8
	pBR1AN	+	-	-	-	-	-	-	-	-	-	3,4,8
	pRme41b:pAK11	N.T.										
	pRmSL26	----------					-	-	-	-	-	8,10
	pNM4AN	+	+	+	+	+	-	-	-	-	-	5

Table 5.1 Continued.

Strain	Plasmid	Nodulation of new host Hac	Inf	Nod	Bar	Fix	Nodulation of normal host Hac	Inf	Nod	Bar	Fix	Source
Rhizobium sp.												
ANU265	pJB5JI	+	+	+	-	-	-	-	-	-	-	5
	pBR1AN	+	+	+	-	-	-	-	-	-	-	5
	pRme41b:pAK11	+	+	D	-	-	-	-	-	-	-	5
	pRmSL26	-	-	-	-	-	-	-	-	-	-	5
	pNM4AN	---	---	---	---	---	+	+	+	+	+	5

Normal hosts: *R.leguminosarum*, peas; *R.trifolii*, white clover; *R.meliloti*, lucerne; *R.spp* NGR234, siratro. D means defective tumour-like nodules which form late.

Sources of data: 1, Hooykaas *et al.* (1981); 2, Van Brussel *et al.* (1982); 3, B. Rolfe, personal communication, and Djordjevic *et al.* (1983); 4, Banfalvi *et al.* (1981); 5, this work and personal observations; 6, S. Long, personal communication; 7, Brewin *et al.* (1980b); 8, J. Denarie, personal communication; 9, Kondorosi *et al.* (1982); 10, Kondorosi *et al.* (1983) [this reference refers to the use of pKSK5, a plasmid essentially the same as pRmSL26]. ANU1004 is a deletion mutant of the *R.meliloti* strain L5-30, and has lost the entire symbiotic gene region including both Nod and Fix functions. In these respects it differs from ZB157, which has only the Hac locus and one Fix locus deleted.

Symbol, --- , means new host does not apply.

Figure 5.1. Visualisation of pNM4AN in the R.meliloti strain ZB157. Tracks are:

A, strain ZB157, which has (a) the megaplasmid pRme41b, which has suffered a deletion in this Nod$^-$ mutant strain, and (b) the cryptic 140 Mdal plasmid pRme41a ;

B, the strain ZB157 (pNM4AN, pJB3JI) which has (a) the megaplasmid pRme41b, (b) the introduced Sym plasmid pNM4AN, (c) the cryptic plasmid pRme41a and (d) the helper plasmid pJB3JI.

This gel was kindly done by Dr. J. Plazinski.

A

B

a
b

a
b
c

d

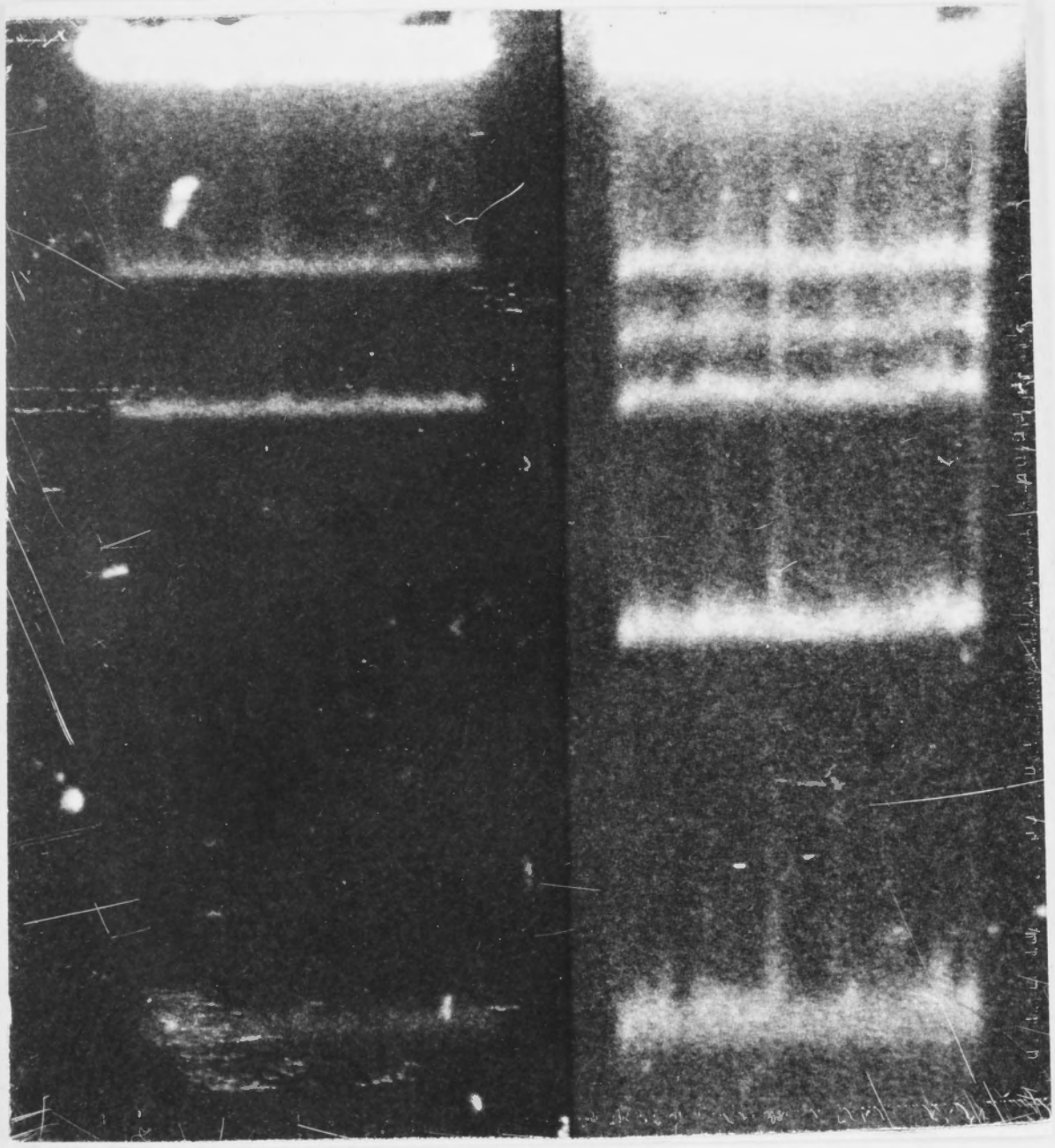


Figure 5.2. Unusual nodules induced by transconjugant strains.

a. Ineffective nodules with "furry" callused surfaces induced on siratro by R.leguminosarum strain 6015 (pNM4AN, pJB3JI). Note that a normal-looking nodule is also present.

b. Typical nodules induced on siratro by either A.tumefaciens strains C58 or A136 carrying pNM4AN. This shows the extensive callusing on the surface of the nodule.

c and d. The defective nodules induced on Leucaena leucocephala by R.meliloti strain ZB157 (pNM4AN, pJB3JI), showing the callused appearence and their proximity to lateral root junctions.

Bar represents 1 mm in each case.


a


b


c

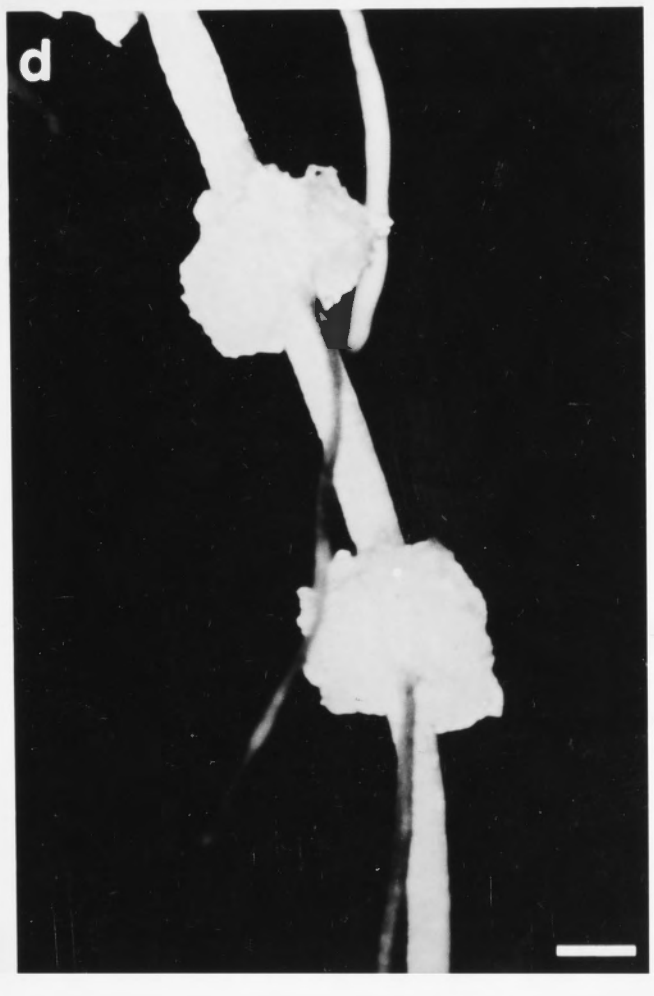
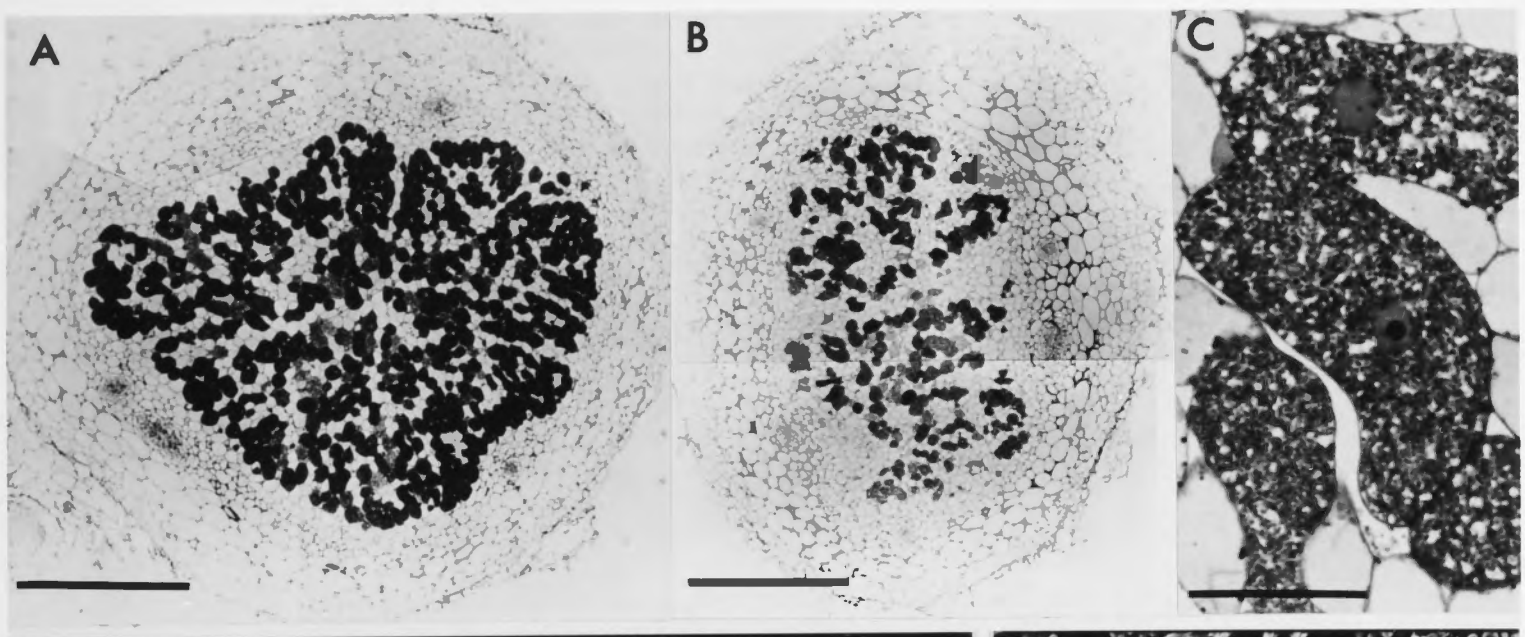

d

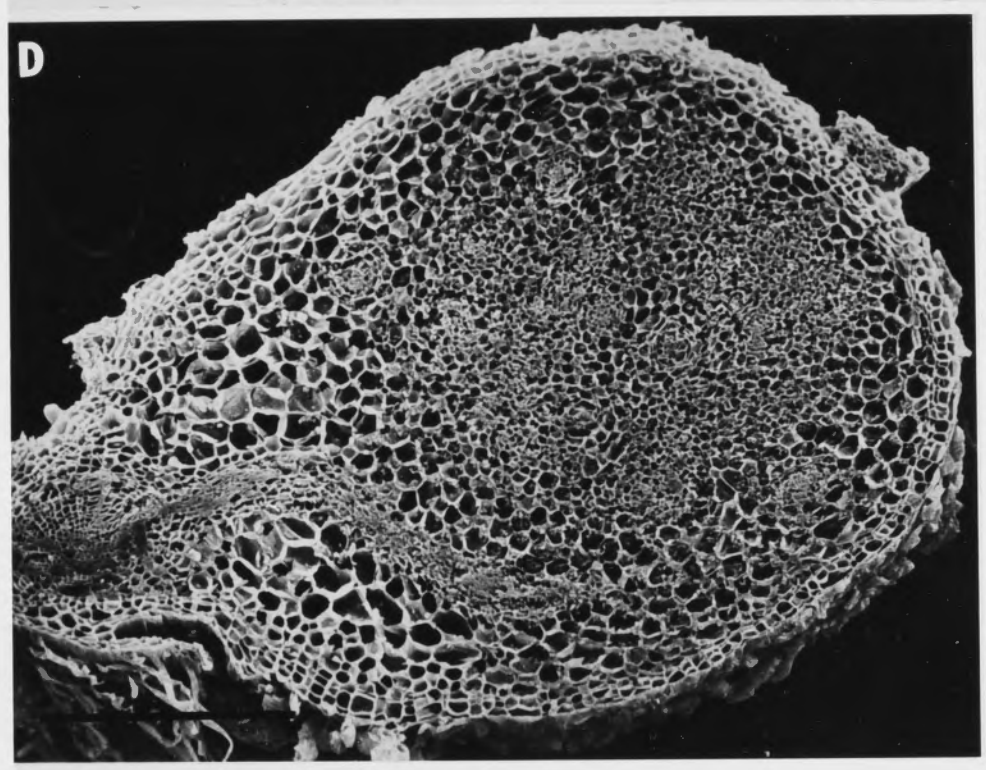
Figure 5.3. Microscopy of siratro nodules induced by various transconjugant strains. A, the normal nodule morphology of siratro, with uninfected cells in the central bacteroid zone, surrounded by the corky cortex of the nodule. This nodule morphology is similar to that of soybean. B, nodule induced by the R.meliloti strain ZB157 (pNM4AN, pJB3JI), showing a similar morphology to the normal nodule. C, R.meliloti ZB157 (pNM4AN, pJB3JI) bacteroids inside the siratro nodule. D, scanning electron microscopy of a nodule induced by R.leguminosarum strain 6015 (pNM4AN, pJB3JI). E, closer examination of the 6015 (pNM4AN, pJB3JI) nodule shows that bacteria have not been released and bacteroids have not developed. F, defective nodule induced by A.tumefaciens strain A136 (pNM4AN, pJB3JI) showing a lack of bacterial infection, and a hyperthrophy of the plant cells.

Bar represents: 0.5 mm in A, B, D and F; 100 microns in C and E.

A, B and F are montages produced by light microscopy.

D and E are scanning electron micrographs.


A
B
C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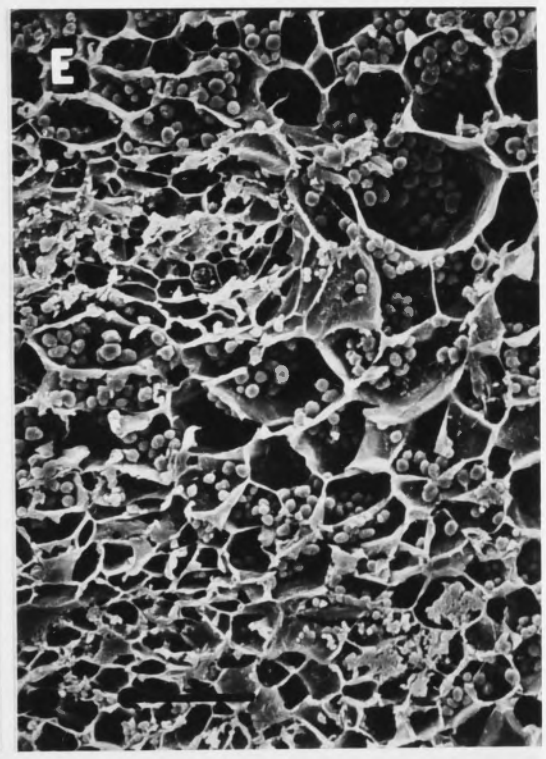
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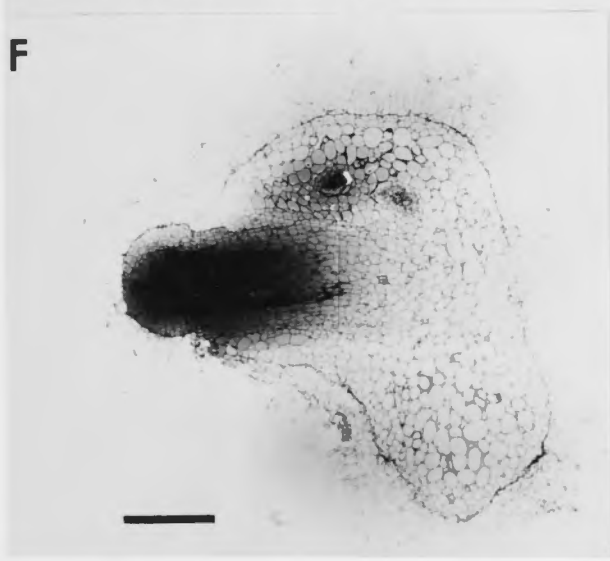

F

CHAPTER SIX

ISOLATION OF SYMBIOTICALLY DEFECTIVE MUTANTS BY TRANSPOSON MUTAGENESIS OF STRAIN NGR234

6.1 INTRODUCTION

Chapters Four and Five showed that the Sym plasmid of NGR234 controls the ability to infect a broad range of plants, and that this trait can be expressed in different bacteria. The control of host-range at the level of early nodulation functions is apparently not influenced by the chromosome or megaplasmid of NGR234, since exogenous Sym plasmids could express early nodulation functions associated with their normal infective host-range, when in the heat-cured strain ANU264. The non Sym plasmid DNA complement appears to be involved in the control of host-specific phenomena after nodule induction, probably at the stage of bacteroid development.

Transposon mutagenesis using Tn*5* has been used to advantage for the isolation of mutants in various *Rhizobium* strains (Beringer *et al.*, 1978a; Cen *et al.*, 1982; Duncan, 1981; Forrai *et al.*, 1983; Meade *et al.*, 1982; Rolfe *et al.*, 1981b). Usually a large range of auxotrophic or symbiotic mutants were isolated, leading to

the conclusion that this transposon has little preference for particular sites as targets for transposition. These authors used the Tn5 mutagenesis system of Beringer et al. (1978a) which utilises the "suicide" plasmid principle (described in Chapter One). This system relies on the fact that a Mu bacteriophage integrated on a plasmid is conditionally lethal in Rhizobium. The exact mechanism is not understood and it is best thought of as a Mu phage -encoded function which either prevents replication or creates a selective pressure to lose the plasmid from the Rhizobium cell. Beringer et al. (1978a) reported that the majority event after mutagenesis of R.leguminosarum was true Tn5 transposition. Meade et al. (1982) showed that both Mu and Tn5 could transpose together in R.meliloti and that deletion mutants of the plasmid occur frequently. For these reasons it is necessary to carefully investigate the behaviour of a suicide mutagenesis system before assuming it will work.

This chapter describes attempts used to apply the system of Tn5 mutagenesis to strain NGR234 in an effort to isolate mutants blocked at the various stages of nodule development. In addition, these experiments show that the original systems developed for Tn5 mutagenesis (Beringer et al., 1978a) are not suitable for use in NGR234 in contrast to the more recent system developed by Simon et al. (1982).

6.2 TRANSFER OF "SUICIDE" PLASMIDS

6.2.1 Transfer of pRK2013

The plasmid pRK2013 carries the Tra genes of RK2, an IncP1 group plasmid, which is essentially identical to RP4 (Burkhardt et al., 1979), cloned onto a derivative of the plasmid ColE1 (Figurski and Helinski, 1979). pRK2013 can transfer at high frequency between E.coli strains but although it can transfer to Rhizobium, it cannot be maintained due to the fact that the IncP1 broad-host-range replication region of RK2 has been replaced by the E.coli-specific ColE1 replication system. Since the Km^R gene of pRK2013 is not transposable, this plasmid is ideal for testing whether non-transpositional "rescue" of Km^R from a suicide plasmid can occur in NGR234, without the complications involved with Mu.

When pRK2013 was mated into ANU239 (a Rif^R strain of NGR234), the rate of Km^R transfer was extremely low, less than 10^{-8}; a rate comparable to spontaneous mutation. This indicates a Km^R gene which is not carried on a transposon cannot be rescued at a detectable frequency in a suicide plasmid system.

6.2.2 Transfer of plasmids containing a phage Mu genome

To assess whether transposon mutagenesis facilitated by Mu phage-containing plasmids could work in NGR234, it was necessary to establish if such plasmids could be

maintained in the Rhizobium strain. This was done by comparing the transfer frequencies of plasmids with and without Mu and Tn5 (the plasmids tested were: RP4, RP4::MuCts::Tn7, pJB4JI and pSP601).

The transfer rates of these four plasmids, using Km^R as a marker, are given in Table 6.1. RP4 transferred at high frequencies and was stably maintained as stated above. The transfer of RP4::MuCts::Tn7 was undetectable, a rate less than 10^{-8}. The Km^R gene on these two plasmids is not encoded on a transposon, so that, in the case of RP4::MuCts::Tn7, no transpositional "rescue" of Km^R can occur. When the suicide plasmids pJB4JI and pSP601 were tested, the frequency of Km^R transfer was 10^{-5}. This value is at least 10^3 times greater than those of RP4::MuCts::Tn7 and pRK2013.

If true suicide mutagenesis was occurring then no coinheritance of other antibiotic resistance markers on the suicide plasmids should occur. This was tested with pSP601. When Km^R alone was used to select for plasmid transfer there was no detectable coinheritance of Tc^R; of 97 Km^R tested none were Tc^R. When Tc^R was used for selection, the transfer frequency was low, about 10^{-6}, and the rate of Km^R coinheritance was high; of 20 Tc^R tested 18 were Km^R. This suggests that the Km^R,Tc^S clones are bona fide transposition derivatives, from events where Tn5 has transposed from the donor plasmid pSP601. Although the Tc^R of pSP601 is encoded on Tn1771, which

theoretically can transpose by itself, Tc^R was usually linked with Km^R. These Tc^R clones therefore must arise from events quite different from true transposition.

Another plasmid-encoded function which can be used in coinheritance testing is the transfer system (Tra). If bona fide Tn5 mutagenesis had occurred then the Tra functions should be lost. However, when putative Tn5 transposition derivatives were spot mated with E.coli strain RR1 it was found that 79 of 155 clones derived from crosses with pJB4JI and 10 out of 30 clones tested from pSP601 matings were able to retransfer Km^R at a high rate to E.coli. Eckhardt plasmid gels on three of these strains, derived from pSP601, revealed plasmids of different sizes. One clone had two plasmids of about 30 and 60 Megadaltons (Mdal). The endogenous Sym plasmid was also seen in all these strains and it was of normal size. One clone, with a single 30 Mdal plasmid, was mated with E.coli on filters and gave a Km^R transfer frequency of about 10^{-3}. The resultant E.coli strain was Km^R but Tc^S, Ap^S and also sensitive to trimethoprim (400 ug ml^{-1} in LBG medium) which are the other markers on pSP601. The 30 Mdal plasmid could transfer at a rate of 10^{-2} to a number of fast- and slow-growing Rhizobium strains and to other E.coli strains. The plasmid was maintained through non-selective subculture and the E.coli strain harbouring this plasmid was sensitive to the donor-specific phages PRD1 and PRR1 which are specific for IncP1 group plasmids

(Stanisch, 1974). It is therefore likely that these small transfer-proficient (Tra^+) plasmids are derived through deletions of the suicide plasmid pSP601.

The clones which are unable to transfer Km^R to E.coli (Tra^-) also had small plasmids. Five clones of this type (derived from a pSP601 mating) were examined by the Eckhardt technique and four of these showed plasmids of various sizes around 40 to 50 Mdal. The donor strain S380 had two plasmids of about 60 and 100 Mdal, which is quite similar to that found with pJB4JI by Kado and Liu (1981). Although this analysis was done on clones derived from matings with pSP601, it is quite likely that the Tra^+ and Tra^- Km^R clones from pJB4JI matings represent the same phenomenon. The formation of deletion derivatives of pJB4JI has been noted in other strains of rhizobia (O'Gara et al., 1982; Simon et al., 1982).

6.2.3 Conclusions on suicide plasmid-mediated mutagenesis

The suicide plasmid pSP601 appears, deceptively, to generate bona fide Tn5 transposition derivatives. The lack of coinheritance of Tc^R with Km^R suggests that the plasmid has been lost in transfer to Rhizobium. The differences in transfer rates of RP4, RP4::MuCts::Tn7, pRK2013 and the suicide plasmids suggests that Tn5 can transpose at a rate of 10^{-3} in this suicide procedure. Although this rate is high in comparison to that found in other Rhizobium species, it is not very different to the

rate of transposition found in E.coli (Berg, 1977). However, the coinheritance of Tra^+ functions and the high number of Tra^- clones which still had plasmids indicates that bona fide transposition is a rare event, even if it occurs at all in the Mu-induced suicide systems. The plasmid pJB4JI appears to behave in a similar fashion.

If the rate of true Tn5 mutagenesis could be calculated it would be less than 10%, given that the only likely candidate found in the above analysis was the single Km^R, Tc^S, Tra^- clone which did not appear to have a deleted suicide plasmid. Whether subsequent problems, such as co-transposition of Mu and Tn5 as described by Meade et al. (1982) and Forrai et al. (1983), could occur in the small margin of bona fide Tn5 mutants generated by pSP601 or pJB4JI was not investigated.

The RP4::MuCts::Tn7 plasmid used in this study was found by Beringer et al. (1978a) to be unable to transfer either RP4 or Tn7-encoded drug resistance to R.leguminosarum. However, O'Gara et al. (1982) found that a similar, independently isolated RP4::MuCts::Tn7 plasmid gave a significant rate of deleted derivatives in R.meliloti.

These data indicate that Tn5 mutagenesis with plasmids pJB4JI and pSP601 does not work in NGR234 as it has in other Rhizobium strains.

6.3 TRANSPOSON MUTAGENESIS WITH THE PLASMID pSUP1011

6.3.1 Transfer of pSUP1011 to strain ANU240

The plasmid pSUP1011 has a gene for Cm^R, a site for mobilisation (Mob) into Gram-negative bacteria, and carries the transposon Tn5 (Simon et al., 1982). The plasmid is derived from pACYC184, a plasmid with a ColE1-like DNA replication system (Chang and Cohen, 1978). This plasmid cannot be maintained in Rhizobium. The Mob site is the Ori-T region of RP4. This Mob region can interact with the transfer functions provided by RP4, in trans, to promote conjugal transfer of whatever molecule contains the Mob site. pSUP1011 can be mobilised between E.coli strains at a frequency of 100% (R. Simon, personal communication), probably because it is a multi-copy plasmid. The E.coli strain carrying pSUP1011, SM10, has an Ap^S, Tc^S, Km^R derivative of RP4, containing a Mu prophage, integrated in the E.coli chromosome. This integrated plasmid provides the necessary transfer functions to mobilise pSUP1011. After pSUP1011 has entered the Rhizobium cell the Tn5 copy can "escape" by transposition while the donor plasmid is lost.

Filter matings with SM10 (pSUP1011) gave a frequency of transfer of Km^R of 10^{-6}. Matings with the donor strain SM10 gave no transfer of Km^R. This control mating was done to ensure that the RP4::Mu(Ap^S,Tc^S) plasmid of SM10 cannot be rescued in strain ANU240.

Two hundred Km^R clones from the mating with SM10 (pSUP1011) were purified and tested for Cm^R, the other marker on pSUP1011. 20% were Cm^R. The Km^R, Cm^S clones are likely candidates for *bona fide* Tn*5* transposition.

The presence of the mobilisation function could be tested by the introduction of an *Inc*P1 plasmid to provide the necessary transfer functions. This test was done in reverse by transferring pSUP1011 to a Rif^R NGR234 derivative (ANU269) which already contained the *Inc*P1 plasmid pJB3JI (a Km^S R68 derivative). The rate of transfer of Km^R was again 10^{-6}. Of two hundred Km^R transconjugants 47% were Cm^R. Those clones that were Km^R Cm^S (106 out of 200) could not transfer either Km^R or Cm^R to *E.coli*. This result shows that the clones that had only Km^R had also lost the Mob site. It also means that no Tn*5* insertions into pJB3JI had occurred.

Transconjugants from the ANU269 test were also selected on Cm and Cm,Km supplemented media. 100 of each type were purified and tested for their ability to transfer Km or Cm to *E.coli*. In each case approximately 80% of the $Km^R Cm^R$ clones were able to transfer Km^R or Cm^R to *E.coli* at detectable frequencies. These results were complicated by variations in transfer rates and loss of expression of Km^R or Cm^R in some instances. The possibility of homologous recombination between the Mob site of pSUP1011 and that of pJB3JI probably accounts for the rise in coinheritance of Cm^R from 20% to 47%. These

factors contribute to make this transfer test complicated in the number of possible interactions. Despite this it is obvious that the Km^R,Cm^S colonies lack any type of mobilisation ability.

Further tests are necessary to be sure that the Tn*5* element is capable of transposing in a random manner. Other workers found that the Tn*1* element had a remarkably high degree of specificity for a few target sites in the entire *R.meliloti* genome (Casadeus *et al.*, 1980). Although Tn*5* has been shown to transpose into many loci in *R.trifolii* (Rolfe *et al.*, 1981b) and *R.meliloti* (Meade *et al.*, 1982; Forrai *et al.*, 1983) it was still possible that it would behave differently in strain NGR234. Obviously, the test of a mutagenesis system is to prove that mutant bacteria with various phenotypes can be isolated and that Tn*5* can be directly associated with the mutation.

6.3.2 Isolation of auxotrophic mutants of ANU240

About 3500 Km^R ANU240 transconjugants from several matings with SM10 (pSUP1011) were purified to single colonies on selective agar. No attempt was made to screen out the Cm^R colonies. Single purified clones were carefully picked and spotted, 50 per plate, on TY medium and TMS medium. TMS medium was made with agar that had been washed overnight with two changes of distilled water to leach out micronutrients.

Thirty five strains were identified as being unable

to grow, or grow only poorly on the TMS minimal medium. These potential auxotrophs were purified again on selective media and attempts made to identify the auxotrophic requirement using the multiple supplement method of Holliday (1956). Likely requirements were thus assigned to most of the strains although some could not be supplemented by any of the pooled nutrients and others had ambiguous requirements. Strains which could not be supplemented could be divided according to whether tryptone or yeast extract alone could support growth. These results are collated in Table 6.2. It can be seen from the table that at least 15 different phenotypes were isolated. A code was used to identify the mutants and this "isolation code" is used here for clarity, although some mutants were later given ANU strain numbers. It should be noted that this work was not intended as a study of auxotrophy in strain NGR234, the analysis of auxotrophs is used purely as a means to judge whether random Tn*5* mutagenesis can occur.

The reversion frequency of a Tn*5*-induced mutation was tested by measuring the rate of recovery of prototrophy in 15 auxotrophic mutants (Table 6.3). Approximately 3×10^9 washed cells of an auxotroph were evenly spread on TMS media and incubated for 5 days. Prototrophic colonies which arose were purified and tested for Km^R. The frequency of reversion was extremely low, in some cases undetectable, in 14 of the 15 auxotrophs

tested. The revertants which were isolated were all Km^S, suggesting that the recovery of prototrophy is associated with the loss of Tn*5*. Only one auxotroph did not follow this pattern. It gave a high reversion frequency (2×10^{-4}) and all revertants of this strain were Km^R.

Some auxotrophs were unable to nodulate, or nodulated in a defective manner (Table 6.4). Auxotrophs with adenine requirements formed callus-like nodules after about five weeks (Fig. 6.1.a). These growths were predominately at the junction of roots, especially in the peripheral roots. Tryptophan-requiring auxotrophs did not nodulate and auxotrophs with uracil requirements were also defective in nodulation, some failing to nodulate at all, whilst others produced late-forming nodules with callused epidermal outgrowths. These tests were done using the plate assay (Rolfe *et al.*,1980) and it is possible that the nodulation response by these strains would change if the plants were grown under different conditions.

Other auxotrophic mutants, even those whose requirement could not be supplemented, were able to nodulate in a seemingly normal manner. This indicates that it is the type of auxotrophy itself which is responsible for the inability to nodulate rather than the trivial explanation that the inoculum cannot grow on F agar.

Some of the auxotrophs used for the reversion analysis were among the defective nodulators. The Km^S

revertants were able to nodulate siratro in a normal manner. This indicates that the Tn*5* was responsible for the auxotrophy and the defective nodulation phenotype.

Although these results are of interest, previous extensive studies on auxotrophy in relation to legume symbiosis had shown much the same kind of result (see Section 6.4). No more work was done using these auxotrophs.

6.3.3 Isolation of symbiotically-defective mutants

The same colonies that were used for the auxotrophic mutant isolation were tested for symbiosis with siratro using the plate method. Small amounts of inocula (estimated at less than 10^8 cells) were used to reduce the possibility of revertants in the inoculum population. To ensure that a maximally effective symbiosis could form, uniform seedlings with a radical of about 1 cm at the time of inoculation were used, followed by incubation in a growth cabinet (see Chapter 2 for specifications) at a temperature of 22^o to 24^oC. This temperature causes slower development of the plant, which results in a more effective symbiosis. After four weeks, obvious Nod^+Fix^+ plant assays were discarded. After eight weeks any presumptive mutants defective in symbiosis, were recorded and parallel repeat plant assays established to verify the mutant phenotype. During the course of the experiment Nod^+Fix^+ strains were constantly culled.

Potential Fix^- mutants were left for up to twelve weeks and some of these eventually showed a Fix^+ response in this time. The assessment of effectiveness relied mainly upon plant vigour and leaf colour since the large number of mutants precluded the use of acetylene reduction assays. Many marginally effective mutants were probably overlooked as a result. However, since I was mainly searching for nodulation mutants this was not considered a critical flaw in the experiment.

Potential mutants sometimes did not give the same phenotype when retested. These pseudo-mutants probably failed to nodulate or fix nitrogen effectively in the initial tests as a result of technical problems such as unhealthy siratro seedlings, contamination by fungi or perhaps positional effects in the growth cabinet. Only those mutants which appeared to have a consistent phenotype were retained. A total of 26 strains were classified according to their response on siratro. The results are summarised in Table 6.4.

Late nodulation mutants: Six strains had delayed nodulation phenotype, taking five to six weeks to nodulate. Although a normal nodule number was produced the association with siratro was ineffective. No further work was done with these strains.

Rare nodulation mutants: Two strains were originally isolated as being Nod$^-$ on siratro. Retests showed that these strains produced an average of one nodule per five plants, rather than the normal number of five to ten per plant. Plants inoculated with these strains appeared Fix$^-$. Acetylene reduction assays were not done.

Ineffective mutants: Ten strains nodulated siratro ineffectively. These strains produced a normal nodule number in the normal time but were unable to produce lush, green siratro plants after 12 to 16 weeks. All had some acetylene reduction activity and the interior of nodules along a plant ranged from white to a faint pink in colour.

Fix$^-$ mutant: Of all the ineffective strains tested only one had a complete lack of acetylene reduction activity. This strain (U7 or ANU1245) formed nodules with a completely white interior.

Defective nodulation mutants: One mutant (L10 or ANU1260) formed callus-like nodule structures rather than normal nodules. These defective nodules had large epidermal outgrowths of a white "furry" appearance. The nodules formed after 6 to 8 weeks mainly in the peripheral roots and at root junctions. The overall appearance of these nodules was the same as those induced by the adenine-requiring auxotrophs described above. In

addition small nodules formed occasionally on the tap root. The mutant could grow on minimal media. This same phenotype was seen in Chapter Five with an Agrobacterium tumefaciens strain harbouring the NGR234 Sym plasmid.

Nod$^-$ mutants: Four mutants were isolated which were unable to form nodules at all on siratro under the conditions of the plate assay. These mutants were studied by Mr. R. Ridge using microscopic observations to assess whether the mutants were capable of root-hair curling on siratro. Only two strains (16-5 and B5-46) were Hac$^-$ on siratro. The other two strains (B7-42 and C5-25) were able to induce root-hair curling and deformities on siratro. Mr. R. Ridge later showed that the mutant B7-42 could nodulate siratro if a high enough inoculum was used in the pouch growth method of Bhuvaneswari et al. (1980). Law et al. (1982) showed a similar result using an R.japonicum mutant with an altered capsule. The mutant C5-25 was also shown by Mr. R. Ridge to grow poorly in unsupplemented liquid minimal medium. It can therefore be considered to be some type of auxotroph.

6.3.4 Characteristics of certain mutants on other plants

It was beyond the scope of this study to investigate the effect of auxotrophic mutations on the host range of NGR234. However selected symbiotic mutants were tested on some other plants. The Hac$^-$Nod$^-$ mutant 16-5 was unable to

nodulate Desmodium intortum, D.uncinatum, L.leucocephala and P.andersonii. The Nod^+Fix^- mutant U7 was Fix^- on D.intortum and L.leucocephala. The mutant L10 which made defective nodules on siratro had a similar but slightly different reponse on D.intortum (Fig. 6.1.b). The nodules were again defective but occurred on the tap root, the development of epidermal callus did not occur, although the nodule surface had a disorganised tumourous appearance. The nodules were obviously blocked at an early stage. Again the A.tumefaciens strain with the NGR234 Sym plasmid (Chapter Five) had a similar phenotype (Fig.6.1.c). Both Hac^+Nod^- mutants (C5-25 and B7-42) were Nod^- on D.intortum.

6.3.5 Hybridisation analysis for the presence of Tn5 sequences

DNA was isolated from a total of 22 mutants, cut with EcoRI and analysed for the presence of Tn5 by Southern hybridisation. An example is shown in Figure 6.2. The probe used, pKan2, contains the internal 3.5kb HindIII fragment of Tn5 cloned into pBR322 (Scott et al., 1982). It contains the entire unique region of Tn5, and a small portion of the left and right inverted repeats (known as IS-50L and IS-50R, respectively). The analysis showed that 19 out of the 22 mutants had a single Tn5 copy. The size range of the Tn5-containing EcoRI-generated fragments was variable but in all cases was

greater than the quoted size of Tn*5*, which is 5.7kb (Meade *et al.*, 1982). This suggests that the Tn*5* of these mutants has different flanking *Rhizobium* DNA, and occurs in a different location in the genome of NGR234. In some cases mutants with the same phenotype had Tn*5*-containing *Eco*RI fragments of a similar size. These mutants could be independent isolations of Tn*5* inserted into the same *Eco*RI fragment or it could be a coincidental occurrence. It is also possible that they are siblings. Three mutants had multiple bands which hybridised to the pKan2 probe (shown in tracks 5, 9 and 12 of Fig. 6.2). The compiled data is presented in Table 6.5.

6.3.6 Presence of the Sym plasmid in Nod$^-$ strains

The mutant strains were analysed by the Eckhardt technique (1978) for the presence of the Sym plasmid. In all but one of the mutants the Sym plasmid was detected and it appeared to be of normal size. The Sym plasmid could not be detected in the Hac$^-$Nod$^-$ mutant B5-46 (ANU1264) although the megaplasmid pRsNGR234*b* was detected. The phenotype of B5-46 can therefore be attributed to the spontaneous loss of the Sym plasmid rather than the insertion of Tn*5*.

6.3.7 Cloning of Tn*5*-containing mutant fragments from some symbiotic mutants

The method used to clone the Tn*5* containing mutated

DNA fragments was to digest about 1 ug of mutant DNA with EcoRI and ligate this into 1 ug of phosphatase-treated EcoRI-cut pBR328 DNA. The ligation mix was then transformed into E.coli RR1 cells and recombinants selected on LB medium containing Km and Tc. Km^R clones were then purified and their plasmid content assayed. EcoRI fragments containing Tn5 were cloned from the mutants B7-42, C5-25, 16-5 and U7 in this manner. The mutated fragments from mutants B5-46 and L10 were cloned and analysed by Judy Tellam. The recombinant plasmids were designated according to their mutant origin, thus mutant B7-42 yielded pB7-42 and so on.

Plasmid DNA was prepared from these recombinants and used as a probe against blots of EcoRI-cut DNA from strain ANU240 and the Sym plasmid-cured strain, ANU264, to determine whether the cloned mutated Rhizobium DNA was derived from the Sym plasmid or elsewhere in the genome. The hybridisation analysis showed that only the Nod^- mutant 16-5 and the Fix^- mutant U7 have Tn5 insertion sites in the Sym plasmid (an example is shown in Fig. 6.3). The size of the wild-type EcoRI-generated DNA fragment from 16-5 was about 2.8kb, and that for U7 about 3kb. These sizes correspond to the predicted sizes of the wild-type fragments, estimated by the size of the mutated fragments. The hybridisation also identified wild-type HindIII fragments; for 16-5 the fragment is about 7kb; for U7 there are two HindIII fragments, one about 3.5kb and

the other about 1.3kb.

The plasmid pU7 was used as a probe against a blot which contained EcoRI digests of DNA from mutants C5-25 and U7 (Fig. 6.4). The probe hybridised to the Tn5-containing fragments present in the DNA as expected. The probe also hybridised to the mutant DNA cloned in pU7. The most important thing to note is that the pU7 probe hybridises to both the mutated fragment (that is, itself) in strain U7 DNA cut with EcoRI, and to an EcoRI fragment which corresponds to the wild-type size of the U7 mutated fragment. This indicates that the wild-type EcoRI fragment of U7, or some part of it, is present in two copies in strain NGR234 and that only one of these fragments is mutated in strain U7. This is a very interesting result since it may indicate that mutating only one copy of a gene, which is apparently duplicated on the Sym plasmid, can lead to a loss of nitrogen fixation activity on both siratro, D.intortum and L.leucocephala. Of course, the mutated gene itself may not be duplicated but the hybridisation could be due to some linked non-symbiotic function which is reiterated. This should be explored in further work.

When mutant 16-5 DNA, cut with EcoRI, was probed with p16-5, only the mutated fragment hybridised. This indicates that the wild-type piece of 16-5 is only present in a single copy on the Sym plasmid, and it is not duplicated. Possible reiteration of the other mutant

fragments was not investigated.

6.4 DISCUSSION

This chapter shows that the newly described Tn*5* mutagenesis system of Simon *et al*. (1982) can be used in the fast-growing cowpea *Rhizobium* strain NGR234. The different phenotypes of mutants isolated and the Southern analysis suggest that Tn*5* has little preference for DNA target sites. The high stability of Tn*5*-induced mutants is reflected by the low reversion rates in 14 of the 15 auxotrophic mutants tested. This work agrees well with work done in *R.meliloti* (Forrai *et al*., 1983; Meade *et al*., 1982). In at least seven auxotrophs, the loss of Tn*5* is correlated with the reversion to prototrophy indicating that the insertion has actually caused the mutant phenotype.

In one auxotrophic strain, the high rate of reversion and the maintenance of Km^R in the revertant population suggests that Tn*5* is not the cause of this mutant phenotype. It is possible that in any strain of bacteria, such as NGR234, endogenous insertion elements will be active in causing mutations. Such elements have been found in *R.meliloti* (Ruvkun *et al*., 1982). If reversion analysis does not show an intimate correlation between Tn*5* and a mutant phenotype, then other methods of verification, such as chromosome mapping and molecular

cloning techniques (Scott *et al.*, 1982), can be employed.

An unexpected result was the high rate of coinheritance of Cm^R and the Mob region in these experiments. One possible explanation is that Tn*5* and other transposons are known to mediate cointegration events between replicons in *E.coli* and other Gram-negative bacteria. Cointegrate structures have been proposed as intermediates in theoretical models of the mechanisms of transposition (Arthur and Sherrat, 1979; Shapiro, 1979). These schemes usually involved two copies of Tn*5* flanking the cointegrated molecule. Therefore, the high rate of Cm^R and Mob coinheritance and the multiple hybridising bands found by Southern analysis (Fig. 6.3) may be different manifestations of the same phenomenon. Simon *et al.* (1982) found no coinheritance of other plasmid markers using pSUP2011 (a plasmid similar to pSUP1011) in *R.meliloti*. This may indicate that Tn*5* transposition is not as efficient in NGR234 as in *R.meliloti*.

Recently Hirschel *et al.* (1982) have shown that Tn*5* can form cointegrate structures at almost the same rate as normal transposition in a recombination-proficient (*recA*$^+$) strain of *E.coli*. In these experiments the result is similar, if the 20% margin of Cm^R, Mob coinheritance represents the rate of cointegration. Since wild-type *Rhizobium* strains are *recA*$^+$, it is not surprising that the rate of cointegration of pSUP1011 is of the same order of magnitude as *bona fide* transposition.

Cointegration of the suicide plasmid pJB4JI has not been reported. If such cointegrates occur, the lethal effects of the Mu phage (cointegrated along with the plasmid) probably prevent their survival. Since pSUP1011 contains no lethal functions there is no selection against cointegrates. Chapter Five described the use of the cointegrative mode of pSUP1011 to form a cointegrate between the NGR234 Sym plasmid and pSUP1011.

Comparison of results in various Rhizobium strains shows that Tn5 is capable of different behaviour in different bacterial species. Meade et al. (1982) could not demonstrate precise excision of Tn5 in a strain of R.meliloti; all but one of twenty auxotrophic strains gave Km^R revertants. They could measure a frequency of transposition of Tn5 (10^{-8}) from a chromosomal loci to a transmissible plasmid. Forrai et al. (1983) had the reverse finding in another strain of R.meliloti; precise excision of Tn5 could occur in auxotrophs, giving Km^S prototrophic revertants, but they could not detect transposition of Tn5 from a chromosomal loci to a transmissible plasmid. In R.trifolii both Km^R and Km^S revertants of auxotrophs can occur and Tn5 is capable of transposition from one locus to another in the Rhizobium genome (B. Bassam, personal communication). In the experiments described in this chapter it is difficult to assess the rate of transposition of Tn5 due to the uncertainty concerning the transfer rate of pSUP1011 to

Rhizobium. However, the frequency of transposition is at least 10^{-6} in the initial mutagenesis experiment.

Some of the symbiotically-defective mutants are similar in phenotype to mutants isolated from other *Rhizobium* strains. This is to be expected if the process of nodulation in siratro is essentially the same as in other legumes. The $Hac^{-}Nod^{-}$ mutant 16-5 (ANU1255) is comparable to Tn*5*-induced mutations of Sym plasmids in *R.trifolii* (the mutants ANU851 and ANU453) and mutants of *R.meliloti* (Forrai *et al.*, 1983; Meade *et al.*, 1982). The defective nodulation mutant L10 (ANU1260) is perhaps similar to two *R.meliloti* mutants (designated 1028 and 1145) described by Hirsch *et al.* (1982) which produce root swellings and gall-like growths on alfalfa roots. Mutants with a similar phenotype have not been described in *R.trifolii* (B. Rolfe, personal communication). The fact that the *A.tumefaciens* strain carrying the NGR234 Sym plasmid had the same phenotype as L10 on siratro and *D.intortum* may indicate that the L10 mutation is in a gene essential for symbiosis which is not found in *Agrobacterium*. This question could be addressed in future work since the L10 gene region has been cloned (J. Tellam, personal communication).

Ineffective and delayed nodulation mutants have been isolated in slow-growing *Parasponia Rhizobium* strains (Cen *et al.*, 1982) and in *R.trifolii* (Scott *et al.*, 1982; Rolfe, personal communication). The mutant C5-25

(ANU1265) is perhaps similar to a $Hac^{+}Nod^{-}$ mutant of R.trifolii, RT3064 (Hughes, 1981). This mutant could initiate a short infection thread in clover root hairs but this thread did not progress down the root hair. RT3064, like C5-25, had a curious unknown nutritional requirement. Although the nodulation phenotypes of these mutants are probably due to pleiotropic effects arising from their growth disabilities, they are still useful in analysing the nodulation process.

Adenine auxotrophy has been linked with a deficiency in symbiosis by a number of authors. In R.leguminosarum (Pain, 1979) and R.meliloti (Scherrer and Denarie, 1971) adenine auxotrophs were ineffective in nitrogen fixation, while Pankhurst and Schwinghamer (1974) describe an adenine-thiamine mutant of R.leguminosarum which was Nod^{-}. Phillips and Torrey (1970) have proposed that adenine is a precursor for cytokinins in Rhizobium and these plant hormones play a role in nodule morphogenesis. Recent findings on tryptophan auxotrophy in R.japonicum (Wells and Kuykendall, 1983) suggest that the formation of indole is essential for symbiosis. Trp^{-} mutants which could synthesize indole were still Nod^{+} whereas mutants affected in indole biosynthesis were Nod^{-}. Since indole is a precursor to the auxin plant hormones it seems obvious to ascribe a role for this hormone in nodule development, especially since Rhizobium strains have been shown to produce auxins (Badenoch-Jones et al., 1982). Both auxins

and cytokinins are involved in the development of tumours induced by Agrobacterium tumefaciens.

In this work the tryptophan auxotrophs were blocked at a very early stage in nodulation while adenine auxotrophs were blocked at later stages. If plant hormones, produced by Rhizobium are really involved in the early nodulation events they are, perhaps, responsible for the initiation of cell division in the root cortex which occurs ahead of the infection thread (Dart, 1977). Cytokinins may be necessary for late nodulation events, perhaps to maintain nodule development, or to effect changes of gene expression in the plant cells. These types of study should be fruitful avenues for future research.

SUMMARY

1. The new Tn*5* mutagenesis system was successful in NGR234.

2. Adenine and tryptophan auxotrophy was associated with defective nodulation responses.

3. Symbiotically-defective mutants of various phenotypes could be isolated. These mutants were similar to mutants found in other *Rhizobium* strains.

4. Only two mutants mapped on the NGR234 Sym plasmid. One had a $Hac^{-}Nod^{-}$ phenotype on all test plants.

Table 6.1 Transfer of suicide plasmids to strain ANU240.

PLASMID	RATE OF Km^R TRANSFER
RP4	10^{-2}
RP4::MuCts::Tn7	$<10^{-8}$
pRK2013	$<10^{-8}$
pSP601	10^{-5}
pJB4JI	10^{-5}
pSUP1011	10^{-6}

Table 6.2 Auxotrophs isolated after Tn5 mutagenesis of strain ANU240 using the plasmid pSUP1011.

ISOLATION CODE	ANU NUMBER	AUXOTROPHIC SUPPLEMENT[1]	NODULATION OF SIRATRO		
			Normal	Late[2]	Defective[3]
Q2	1200	ade	-	+	+
U6	1201	ade	-	+	+
1-36	1210	ade-ura	-	+	+
4-40	1211	ade-ura	-	+	+
19-45	1216	asp	+		
20-42	1217	asp	+		
13-21	1220	asp-glu	+		
13-28	1221	asp-glu	+		
5-39	1218	asp-glu	+		
8-38	1219	asp-glu	+		
M3	1222	arg	+		
Z1-7	1223	arg	+		
10-21	1214	cys	+		
40-1	1215	cys	+		
A7-43	1227	iso-val	+		
Z8-4	1226	iso-val	-	+	
21-43	1228	iso-val	+		
8-44	1224	leu	+		
11-24	1212	met	+		
Q-10	1213	met-cys	+		

Table 6.2 continued.

ISOLATION CODE	ANU NUMBER	AUXOTROPHIC SUPPLEMENT[1]	NODULATION OF SIRATRO		
			Normal	Late[2]	Defective[3]
A3-7	1207	trp	-	-	-
A3-41	1208	trp	-	-	-
C2-21	1209	trp	-	-	-
Z6-8	1206	trp	-	-	-
1-22*	1225	tyr	+		
B2-37	1205	ura	-	-	-
B7-49	1204	ura	-	-	-
P6	1203	ura	-	+	+
24	1229	tryptone	+		
W5	1230	poor on TY	+		
C4-21	1231	yeast extract	+		
40-3	1232	TY only	+		
40-4	1233	TY only	-	+	+
40-5	1234	TY only	-	-	-

Note

1. Abbreviations are: ade - adenine; asp - aspartic acid; arg - arginine; cys - cysteine; glu - glutamic acid; iso-val - isoleucine and valine; leu - leucine; met - methionine; trp - tryptophan; tyr - tyrosine; ura - uracil.

2. Late nodulation occurred after six weeks.

3. Defective nodulation: Callus-like nodules with furry, white epidermal outgrowths, occur mostly on lateral roots, ineffective symbiosis.

4. *; This mutant was derived from ANU269.

Table 6.3 Characteristics of mutants tested for reversion to prototrophy.

ISOLATION CODE	ANU NUMBER	AUXOTROPHY	RATE OF REVERSION	NUMBER ISOLATED
A. Mutants which gave revertants				
Q2	1200	ade	10^{-9}	1
U6	1201	ade	10^{-9}	3
P6	1203	ura	10^{-9}	1
A3-41	1208	trp	10^{-9}	1
C2-21	1209	trp	10^{-9}	1
Q10	1213	met-cys	10^{-9}	1
Z8-4	1226	iso-val	10^{-9}	1
M3	1222	arg	2×10^{-4}	10^{5}
B. Mutants which did not give revertants				
B7-49	1204	ura		
B2-37	1205	ura		
Z6-8	1206	trp		
A3-7	1207	trp		
A7-43	1227	iso-val		
24	1229	unknown		
W5	1230	unknown		

Table 6.4 Symbiotically defective mutants isolated after Tn*5* mutagenesis.

ISOLATION CODE	ANU NUMBER	SYMBIOTIC PHENOTYPE[1]	Tn*5* PIECE (kb)[2]	LOCATION OF Tn*5* [3]
16-5	1255	$Hac^{-}Nod^{-}$	8.5	Sym plasmid
B5-46	1264	$Hac^{-}Nod^{-}$	15	chromosome
B7-42	1266	$Hac^{+}Nod^{-}$	13	chromosome
C5-25	1265	$Hac^{+}Nod^{-}$	7.0	chromosome
C6-13	1262	$Hac^{+}Nod^{R}$	6.5	N.D.
A5-33	1263	$Hac^{+}Nod^{R}$	N.D.	N.D.
L10	1260	$Hac^{+}Nod^{D}$	15	chromosome
U7	1245	$Nod^{+}Fix^{-}$	9.0	Sym plasmid
10 strains		$Nod^{+}Fix^{I}$	N.D.	N.D.
6 strains		Nodule formation delayed	N.D.	N.D.

Note to Table 6.4

1. Hac refers to siratro root hair curling. Nod^- means lack of any nodules or root distortions on inoculated plants. Nod^R means nodules rarely formed. Nod^+ means normal nodulation. Nod^D means defective nodulation. Fix^- means a lack of nitrogen fixation. Fix^I means an ineffective response but with acetylene reduction activity. N.D. means not determined.

2. Tn*5* piece refers to the size, in kb, of the Tn*5*-containing EcoRI fragment.

3. Chromosome means both the chromosome and the megaplasmid pRspNGR234*b*.

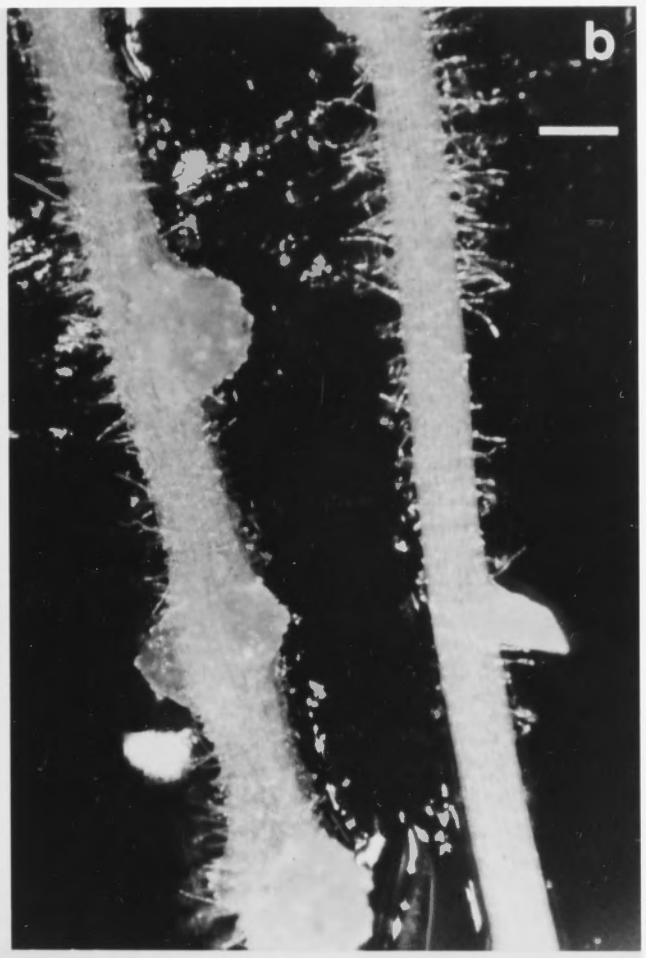
Hair curling data was kindly provided by Mr. R. Ridge.

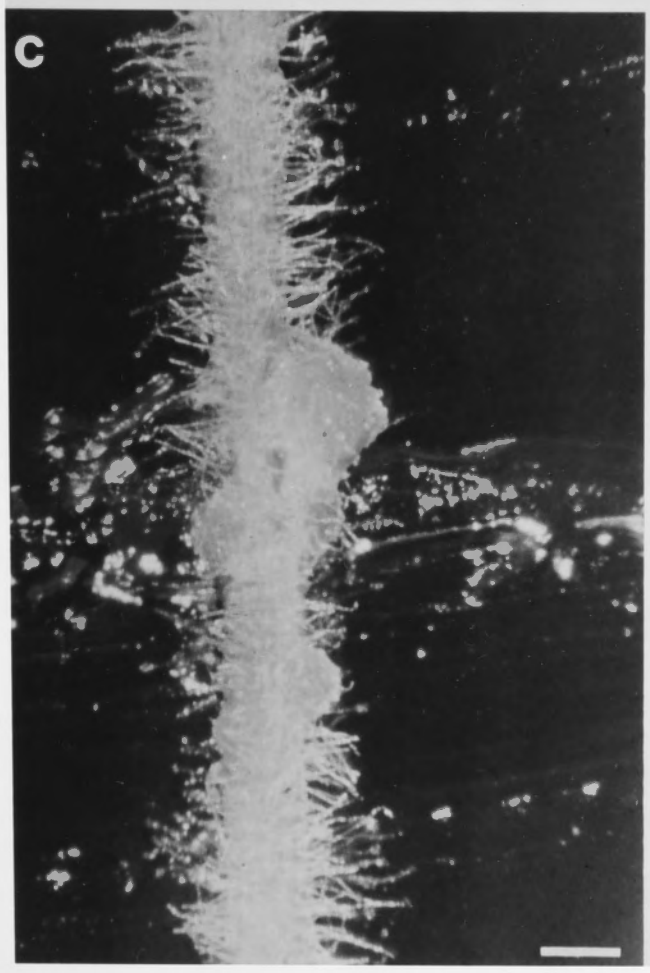
Table 6.5 Summary of the Southern hybridisation analysis for the presence of Tn*5* in ANU240 mutants.

ISOLATION CODE	PHENOTYPE	NUMBER OF BANDS HYBRIDISING	SIZE OF THE *ECO*RI BAND(kb)
Q2	Ade^-	1	8
U6	Ade^-	1	9
A3-7	Trp^-	1	15
C2-21	Trp^-	1	15
B7-49	Ura^-	1	11
P6	Ura^-	1	6
C4-21	Auxotroph	1	13
C5-25	Nod^-	1	7
B5-46	Nod^-	1	20
B7-42	Nod^-	1	12
16-5	Nod^-	1	8
C6-13	Nod^R	1	6
L10	Nod^D	1	12
A3-40	Delayed Nod	2	9, 6
B5-42	Delayed Nod	3	16, 11, 7
A5-28	Ineffective	2	18, 8
A10	Ineffective	1	8
B1-30	Ineffective	1	10
G-4	Ineffective	1	7.5
K-4	Ineffective	1	10
L-5	Ineffective	1	10
U7	Nod^+Fix^-	1	9

Figure 6.1. Defective nodulation phenotypes. a; The "furry" defective nodulation phenotype. This occurs on siratro with some late nodulation mutants, mutants auxotrophic for adenine or uracil as described in Table 6.2, and the defective nodulation mutant L10. It also occurs with the A.tumefaciens strain A136 (pNM4AN, pJB3JI) described in Chapter 5. b; Nodules blocked in an early stage of development on Desmodium intortum inoculated with the mutant L10. c; Again, the strain A136 (pNM4AN, pJB3JI) has a similar phenotype to the mutant L10. Defective nodules on D.intortum. d; Roots of D.intortum inoculated with the Hac^- mutant 16-5. Bar represents 1 mm in each case.


a


b


c


d

Figure 6.2. Detection of Tn5 by specific hybridisation in 11 presumptive Tn5 induced mutants. This hybridisation is an example of the analysis done on 22 mutants. Track 1 contains molecular weight markers, 1 ug of phage lambda DNA cut with HindIII. Track 2 is strain ANU240 included as a negative control for the presence of hybridisation of the pKan2 probe to the wild-type DNA. Track 3 is strain ANU264 (pJB5JI) used as a positive control for hybridisation to Tn5. In tracks 5 and 12 are two mutants which have double hybridising bands. Track 9 is a late nodulation mutant which is Cm^R and has three bands which hybridise to the pKan2 probe. The other seven tracks are various auxotrophic and symbiotically defective strains, all of which have a single copy of Tn5. The faint bands in tracks 6 and 10 are due to partial digestion of the DNA by EcoRI. Differences between tracks in intensity of the bands are due to differences in the amount of DNA run for each mutant, which was 2 to 3 ug per track. The lambda marker fragments were visualised by probing the filter with radioactively labelled lambda DNA, after the hybridisation with pKan2 was completed. In this case, the degree of hybridisation is proportional to the molecular weight of a marker fragment, since the entire lambda molecule was used as a probe.

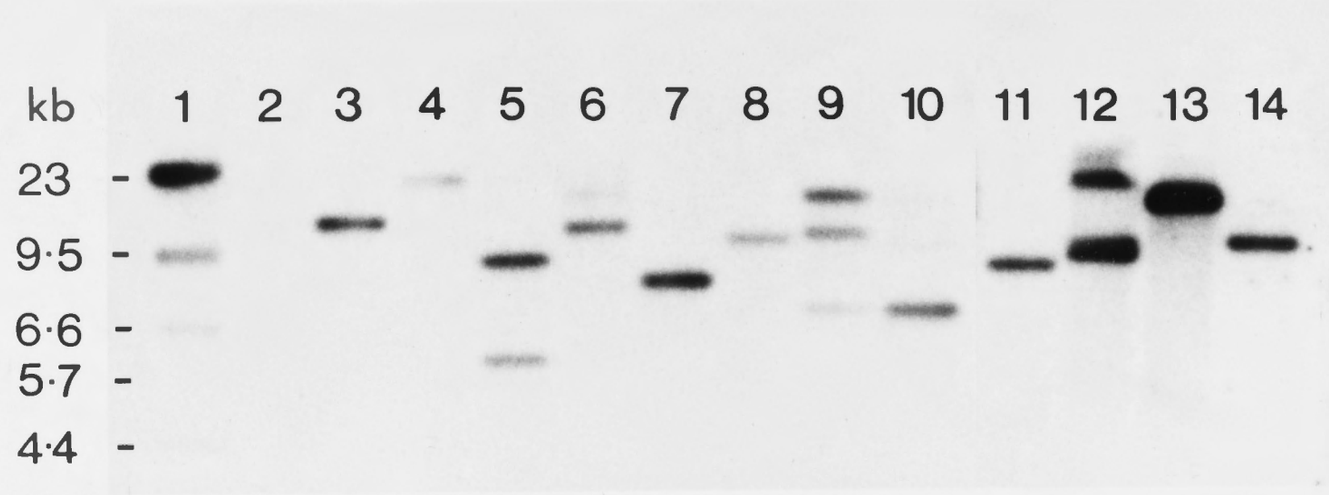

kb
1
2
3
4
5
6
7
8
9
10
11
12
13
14
23
9·5
6·6
5·7
4·4

Figure 6.3. This blot is an example of the analysis used to determine if a Tn5 insertion is on the Sym plasmid or in the "chromosome" (the chromosome and megaplasmid) of strain NGR234. The probe used was the plasmid pU7, a recombinant pBR328 derivative containing the Tn5 and flanking Rhizobium DNA from the Fix$^-$ mutant U7. The strains used to make the DNA used in the blot are: track 1, ANU240; track 2, ANU264 (the heat-cured strain); track 3, ANU264 (pJB5JI); track 4, ANU264 (pBR1AN). The bands occurring in tracks 3 and 4 are characteristic of the Tn5 copies found on pJB5JI and pBR1AN. Strain ANU264 does not hybridise at all to the probe, while strain ANU240 shows an EcoRI band of about 3kb. This size corresponds to the predicted size of the wild-type fragment for the mutant U7, since the Tn5-containing fragment cloned in pU7 is about 9kb. Since the only difference between ANU240 and ANU264 is the lack of the Sym plasmid in ANU264, this analysis shows that the fragment cloned from the mutant U7 was derived from the Sym plasmid. When mutants with Tn5 insertions not in the Sym plasmid are analysed (not shown), hybridisation occurs to all DNA in this system.

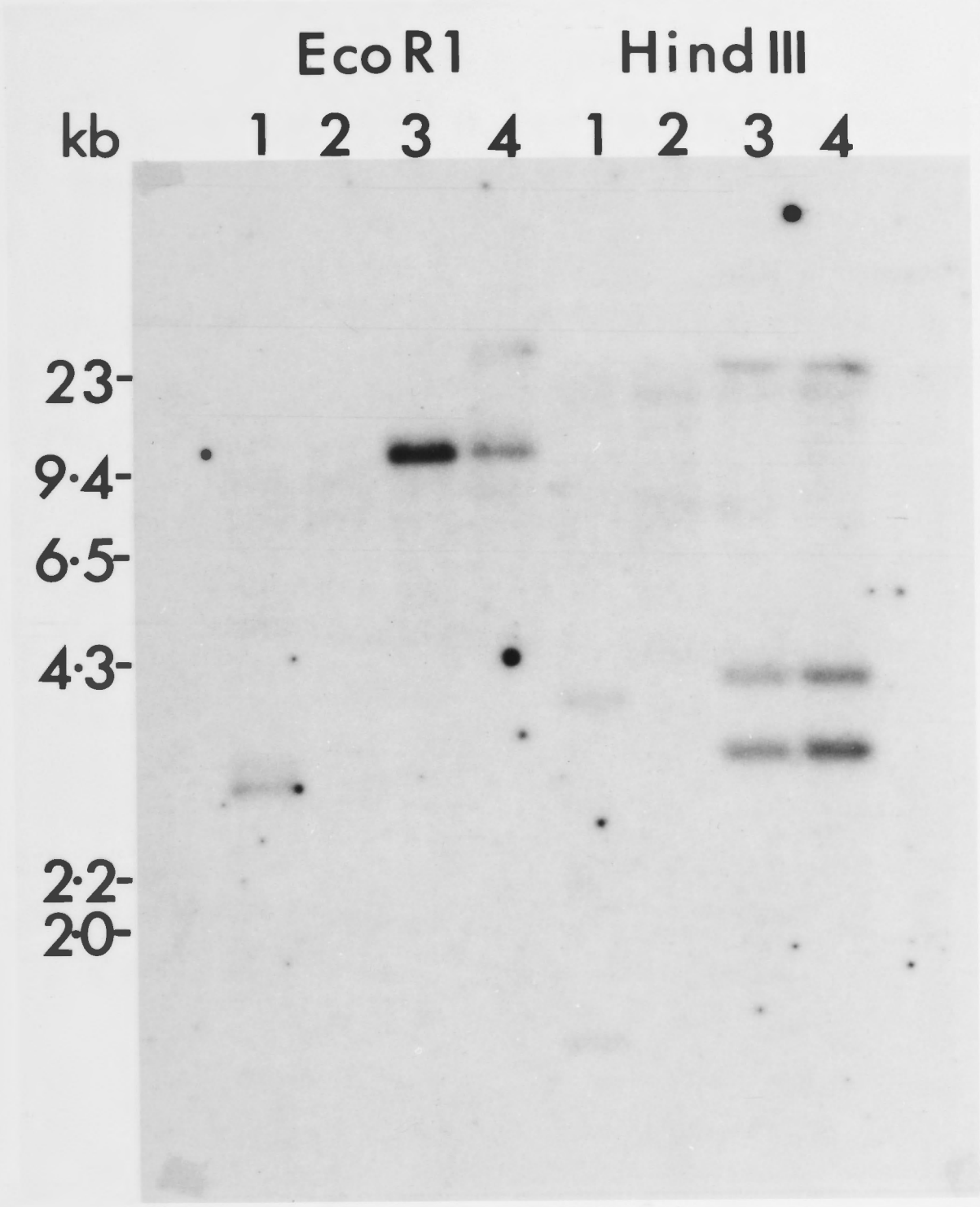
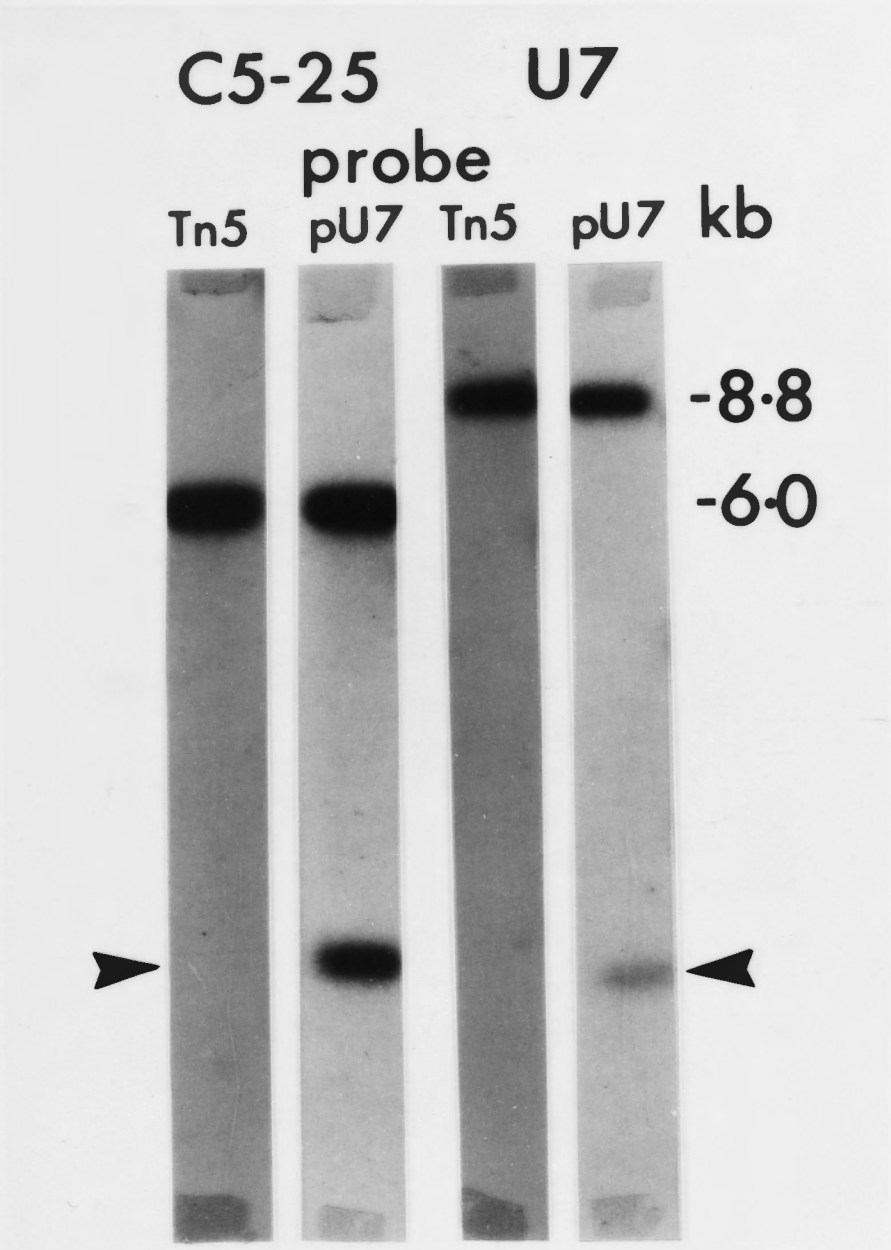

EcoR1
HindIII
kb
1 2 3 4 1 2 3 4
23-
9·4-
6·5-
4·3-
2·2-
2·0-

Figure 6.4. This figure shows that some DNA associated with the region cloned in the plasmid pU7 is duplicated. The mutants C5-25 and U7 were used for this analysis. C5-25 is a Hac$^+$ Nod$^-$ mutant, described in the text, and was used as a control for the presence of both Tn*5* and the wild-type sequences from the U7 region. U7 is a Fix$^-$ mutant which has Tn*5* inserted in the Sym plasmid. The DNA was digested with *Eco*RI and blotted as described in Chapter Two. The tracks shown were on the same blot and the figure has been spliced together to remove irrelevant digests. The blot was first probed with the Tn*5* probe, pKan-2 (which has been shown to have no homology with NGR234 total DNA) to produce the image indicated. In C5-25 only the fragment corresponding to the Tn*5* insert is hybridising. Likewise, a Tn*5* probe on U7 DNA only identifies the fragment with the Tn*5* insert. The sizes of the Tn*5*-containing fragments are different in C5-25 and U7 because they are in different sites in the genome. The other probe used, pU7, is the pBR328 recombinant which contains the Tn*5* and flanking *Rhizobium* DNA from the mutant U7. When this plasmid was probed against C5-25 DNA, again the Tn*5* copy hybridises, and so does the unmutated "U7" fragment which is about 3kb (shown by the arrow). In the mutant U7, one would expect only the mutated, Tn*5*-containing fragment to hybridise to the pU7 probe (i.e. the cloned fragment hybridising to itself). However, the pU7 probe hybridises to an *Eco*RI piece of the same size as the unmutated "U7" fragment identified in C5-25 and Figure 6.3. This could mean that the "U7" fragment is duplicated, and that Tn*5* is inserted in only one of the copies, leaving the other unmutated. Alternately, the cloned DNA could have a repeated element which is only present in two copies and both copies are fortuitously on 3kb *Eco*RI fragments.


C5-25
U7
probe
Tn5
pU7
Tn5
pU7
kb
-8·8
-6·0

CHAPTER SEVEN

MOLECULAR CLONING OF GENES ESSENTIAL FOR THE FIRST STEP IN SYMBIOSIS: PLANT ROOT HAIR CURLING

7.1 INTRODUCTION

Chapter Six described the isolation of various mutant derivatives of strain ANU240 which are unable to form a normal symbiosis with siratro. Each of these mutant strains is interesting in its own right and could be pursued further. However, it was decided to concentrate only on the nodulation mutant which mapped on the NGR234 Sym plasmid. The Hac$^-$ Nod$^-$ mutant 16-5 (ANU1255) was unable to nodulate a number of plants including Parasponia. Whatever gene is mutated in 16-5 is absolutely necessary for nodulation by root hair invasion and whatever mode occurs on Parasponia.

In Rhizobium trifolii it had already been shown that genes for nodulation and host-specificity could be cloned intact on a single large DNA fragment derived from the Sym plasmid of strain SU843 (Schofield et al., 1983b). The work in Chapter Five showed that the NGR234 Sym plasmid carries genes responsible for the infective host-range of NGR234. It was hoped that a close linkage would exist on the Sym plasmid between a Nod$^-$ mutant and genes for

host-range, and that a fragment similar to the Rhizobium trifolii "Nod" fragment (a 14kb HindIII piece) could be cloned from strain NGR234.

This chapter describes the cloning of the DNA region flanking the mutation in 16-5. These cloned DNA fragments were used to investigate the organisation of nodulation genes in NGR234, and the conservation of early nodulation genes between NGR234 and R.trifolii.

7.2 ISOLATION OF THE WILD-TYPE NODULATION GENE REGION

7.2.1 Recombinant lambda bacteriophages

The Tn5 containing fragment, generated by an EcoR1 digest of total DNA from 16-5, was cloned into pBR328 to give the plasmid p16-5 (described in Chapter Six). This plasmid was used as a probe to isolate nine independent lambda recombinants, from a lambda Charon-28 gene bank of ANU240 as detailed in Chapter Two. DNA of these phages was digested with various restriction endonucleases and maps constructed. The phages were shown to overlap as displayed in Fig.7.1. Some phages were identical so only the unique phages are shown in the map. To verify that the DNA fragments in the recombinants are actually found in the genome, each phage was used as a probe on digests of total genomic ANU240 DNA cut with a number of restriction endonucleases (an example is shown in Fig. 7.2). Three phages were "monsters" which had a portion of

the "Nod" region, but had extra fragments which could not be rationalised with the other phages and the fragments demonstrated to occur in the genome. Lambda 16, shown in Fig. 7.2 is an example of a "monster" phage. This phage has an insert of about 16kb, but only hybridises to a small portion of the "Nod" region. The origin of the other DNA in this phage is unknown.

7.2.2 Isolation of wild-type HindIII fragments

To further verify, and characterise the DNA region flanking the mutation in 16-5, wild-type DNA fragments were isolated from another gene bank. A HindIII clone bank was constructed by ligating 2 ug of HindIII digested total ANU240 DNA onto 2 ug of HindIII cut phosphatase treated pBR322 DNA. About 10^4 E.coli transformants were generated, and about 3000 of these were screened by colony hybridisation using lambda 20 DNA as a probe. Positively hybridising colonies were chosen, purified and screened for plasmid content. Fragments cloned were a 6.7kb HindIII fragment (which occurred in lambda 3), a 9.4kb fragment (which occurred in lambda 5, 6 and 20) and a 24kb fragment, mentioned later, which contains a repeated DNA region. The 6.7kb HindIII fragment was isolated three times in the clone bank and each clone also contained a 2.3kb HindIII fragment. This extra fragment was shown to exist adjacent to the 6.7kb fragment in the genome, when an overlapping fragment was cloned. The pBR322

recombinant containing both the 6.7 and 2.3kb HindIII fragments was called pRs1.

The 6.7kb fragment was purified by electroelution and used to probe a blot of genomic ANU240 DNA cut with a number of restriction enzymes. The result (Fig. 7.3) confirmed previous hybridisations done using p16-5 and verified the map. This hybridisation also gave further information on the restriction enzyme sites in the 6.7kb fragment, and showed that this fragment is unique in the genome, and is free of repeated sequences.

7.2.3 Cloning of a large XhoI fragment

A large Xho I fragment of about 16kb was identified by the hybridisation data. This fragment offered the opportunity of cloning a large part of the "Nod" region on a single piece of DNA. Other larger fragments (generated by Kpn I and Sma I) exist but these were considered impossible to clone due to a lack of appropriate vectors. Several unsuccessful attempts were made to clone the XhoI fragment from total XhoI clone banks of ANU240 made in various vectors. Finally two methods were used to purify large sized XhoI fragments from XhoI digested total DNA. Both these methods lead to the cloning of the XhoI fragment.

In the first method approximately 200 ug of total genomic ANU240 DNA was cut to completion by XhoI and resolved overnight in an 1.0% low melting agarose gel. A

gel slice was taken which contained DNA of sizes 9 and 23kb, and the DNA was purified by electroelution, phenol-chloroform extraction and ethanol precipitation (Chapter Two). Approximately 2 ug of DNA was recovered from this procedure. The second method utilised a linear 5 to 25% sodium chloride gradient in TE buffer. About 200 ug of total genomic DNA was cut with XhoI to completion and loaded onto a gradient prepared in a Beckman SW-27 tube. The gradient was spun at 25,000 rpm for 4hr at 15°C in a Beckman SW-27 rotor. Fractions of 0.5 ml were taken by pumping out the gradient at a flow rate of 50 ml per hour. Of every third fraction, 10 ul was assayed on a gel and those fractions having the best separation of the large XhoI fragments were pooled and dialysed against TE buffer overnight. The dialysate was concentrated from about 5ml to 0.5ml by repeated equilibration against butanol. The DNA was precipitated and redissolved in 20 ul of TE. About 0.5 ug was recovered.

Approximately 0.5 ug of DNA from both procedures was ligated to 1 ug of XhoI cut phosphatase-treated pACYC177 DNA using 10^4 units of T4 DNA ligase and standard conditions (see Chapter Two). One tenth of each ligation was transformed into E.coli RR1, yielding about 100 Ap^R transformants each. These colonies were extracted onto nitrocellulose and probed with nick-translated pure 6.7kb HindIII fragment DNA. Each ligation gave about 5 positive colonies. Only one was chosen for further work. This

plasmid was designated pRs50. Restriction mapping of pRs50 confirmed the previously deduced fragment order.

A reiterated sequence is present in the DNA region cloned. This sequence is obvious in hybridisations done with the 16kb XhoI fragment, various lambda recombinants, and other DNA fragments. For example, the DNA of lambda 20 (a "real" phage) hybridises to more DNA in the genomic blot than can be accommodated in lambda Charon-28 (Fig. 7.2). Lambda 20 hybridises to HindIII bands of 24, 23, 16, 12, 9.4, 6.7, 2 and 1.5kb. This totals 84kb. The only bands derived directly from lambda 20 are the 9.4, 6.7 and 2kb. The repeated DNA region appears to be present in at least 5 copies. The copy in the "Nod" region was given an approximate position by comparing hybridisations done with various sub-clones of the DNA region (Fig. 7.1). Further work is needed to fully characterise this repeated DNA region.

7.3 CIRCUIT EXPERIMENT

To prove that the Tn5 insertion in the Hac$^-$ Nod$^-$ mutant 16-5 is really the cause of the mutant phenotype it is necessary to reintroduce a wild-type fragment on some appropriate vector to assay for a return to nodulation ability. This experiment was done using the 6.7kb HindIII fragment. The 6.7kb fragment was re-cloned from pRs1 onto the vector pSUP202, and the resulting plasmid designated

pRs23. pSUP202 is derived from the E.coli cloning vector pBR325 and cannot replicate in Rhizobium. For the plasmid markers to be inherited, the plasmid must be rescued by some kind of recombinational event. The plasmid pJB3JI (a Km^S derivative of RP4) was used to mobilise pRs23 in a triparental patch mating to strain ANU1256 (a Sp^R mutant of 16-5) and to strain ANU265 (the heat-cured Nod^- mutant of NGR234). Cm at 15 ug ml^{-1} was used in TM medium to select for transconjugants. The rate of transfer of Cm^R to ANU1256 was about 10^{-4} for pRs23. With ANU265 as a recipient, pRs23 gave only a few Cm^R colonies.

Both siratro and Desmodium intortum plants were inoculated directly with unpurified ANU1256 (pRs23) transconjugants since neither donor nor recipient strains can form nodules. After 10 days visible nodules had developed in a profuse manner on all plants inoculated with ANU1256 (pRs23). Plants inoculated with strains ANU240 and NGR234 had all nodulated, and uninoculated controls were all Nod^-. ANU265 were unable to nodulate either siratro or D.intortum. Plants nodulated by the corrected mutant eventually gave a Fix^+ response after 2 months.

Siratro root hair curling was assayed after two weeks by direct observation of roots in agar using a binocular microscope. Strains ANU240 and ANU1256 (pRs23) were able to induce root hair curling and root hair distortions whilst strains ANU1256 and ANU265 were unable to cause

distortions or root hair curling.

In an effort to analyse how the phenotypic correction of ANU1256 occurred, bacteria were isolated from nodules of siratro and D.intortum and tested for the presence of Tn5 (Km^R). All eight nodule extracts derived from ANU1256 (pRs23) retained Km^R. Total DNA was made from a number of nodule isolates to further investigate what changes had happened at the DNA level. Southern hybridisation to total DNA of the corrected mutants (cut with HindIII) using pRs23 DNA as a probe showed that the Km^R ANU1256 (pRs23) strains have the wild-type 6.7kb HindIII fragment, the vector plasmid band of 8.5kb (pSUP202) and two bands characteristic of the arrangement of fragments in the mutant ANU1256 (Fig. 7.4). This means that both the mutated and wild-type fragments are present. The intensity of hybridisation suggests an equal copy number of the fragments. The simplest explanation for this arrangement is to assume that pRs23 has been rescued by a cross-over event within the 6.7kb HindIII fragment. A single cross-over does not remove the Tn5 and results in the insertion of pSUP202 and the wild-type 6.7kb HindIII fragment into the Sym plasmid. This accounts for the equal copy number since the inserted DNA is replicated with the Sym plasmid.

A Southern analysis using XhoI instead of HindIII can distinguish between insertion of the pRs23 plasmid in the Nod region or some other locus. XhoI cuts Tn5 but does

not cut pRs23. The Tn5 in the mutant is near the centre of a 16kb XhoI fragment so a recombination between pRs23 and this fragment would result in an alteration in size of the XhoI fragments. If pRs23 is inserted elsewhere by some other recombinational event the pattern of XhoI fragments will be unaltered. Figure 7.5 shows the result of analysing four corrected mutants. The probe used in this experiment was the entire 16kb XhoI fragment, so extra hybridising bands are present due to the repeated sequence on this DNA fragment. However the control tracks containing ANU240 and the mutant 16-5 DNA clearly show that the repeated sequence hybridisation bands are unchanged in all strains. The 16kb XhoI is split into two pieces of about 8 and 9kb in the mutant 16-5. In two cases pRs23 has recombined in the 8kb piece and in the other two it has recombined in the 9kb piece. This result shows that correction can occur by recombination on either side of the Tn5.

This result means that the 6.7kb HindIII fragment has one entire transcriptional unit, perhaps an operon. This can be reasoned simply by considering the possible arrangements of genes in this region (Fig 7.6). If the operon containing the 16-5 "gene" started outside one of the HindIII sites, proceeded through the 6.7kb HindIII fragment and terminated beyond the other HindIII site, the only possible means of correction would be by double cross-over (cis complementation). If the operon started

outside the 6.7kb HindIII piece, went through the 16-5 "gene" and terminated before the second HindIII site then correction would be possible by single cross-over, but only on the side of the Tn5 closest to the promoter sequence controlling transcription. If the promotor is actually within the cloned fragment and the operon continues beyond the end of the HindIII site then correction can occur by single cross-over, but again only on one side of the Tn5. The only way that correction can occur equally by cross-over on either side of the Tn5 is if the entire operon is on the cloned DNA. The direction of transcription of the Hac genes cannot be deduced from this experiment.

Since the 6.7kb HindIII fragment apparently has an entire transcriptional unit, it was obvious to test if this fragment contained all the genes required for either root hair curling or nodulation. This was done by re-cloning the fragment onto the vector pSUP106, which is derived from the IncQ group plasmid RSF1010 and can replicate in Rhizobium (Simon et al., 1983). The pSUP106 recombinant containing the 6.7kb HindIII fragment was called pRs21. This plasmid was mobilised into ANU265 using the helper plasmid pJB3JI, selecting for Cm^R, the only marker on pRs21. Strain ANU265 (pRs21) was unable to cause root hair curling or distortions and was unable to nodulate siratro. This indicates that the 6.7kb fragment does not have all the genes required for either the early

nodulation event of root hair curling or for nodulation.

The plasmid pRs21 was mobilised into the Hac^- mutant ANU1256 in the same manner as described above for pRs23. ANU1256 (pRs21) was able to nodulate siratro promptly and eventually showed a Fix^+ response. Some nodule reisolates (3 out of 9) had lost both Km^R and Cm^R, suggesting that marker exchange, or double reciprocal recombination, happened. Southern hybridisation (Fig. 7.4 and Fig. 7.5), analysis confirmed that these strains had perfectly reconstituted 6.7kb HindIII and 16kb XhoI fragments. This is consistent with double reciprocal recombination.

Since the 6.7kb HindIII fragment did not contain all Nod or Hac genes, it was hoped that the large XhoI fragment identified previously would contain all the Sym plasmid encoded nodulation genes from ANU240. From the plasmid and lambda recombinant mapping data, the end of this XhoI fragment could be put about 8kb to the left of the site of the Tn5 insertion in 16-5. As the XhoI fragment is about 16kb long the location of at least one Nod gene is in the center of this fragment.

The 16kb XhoI fragment was sub-cloned from pRs50 into pSUP106, a vector capable of replication in Rhizobium. About 1 ug of pSUP106 was digested to completion with SalI (which cuts in the Tc^R gene of this vector) and ligated in the presence of 1 ug of XhoI cut pRs50 DNA. The ligation was done at 16^oC for 16hr. Transformants were selected on LB agar with Cm and subsequently screened for Tc^S. Eight

Cm^R, Tc^S transformants were screened for plasmid content and only one had the pattern predicted for pSUP106 carrying the 16kb XhoI fragment cloned in the SalI site. The plasmid was designated pRs51. The mobilising strain SM10 was transformed with pRs51 and the resulting strain (called SM10 (pRs51)) was used as a donor in a patch mating with strain ANU265 on TY medium. Transconjugants were selected on TM medium with Cm. After seven days Cm^R ANU265 transconjugants had grown on the selective plates. ANU265 (pRs51) cells were used to inoculate siratro and Leucaena leucocephala plants. No nodules developed on any of 60 test plants and no root hair curling could be seen on repeated inspection over two months.

These results suggest that the 16kb XhoI fragment does not have all the genetic requirements for root hair curling or nodulation. This argues that the arrangement of nodulation genes in NGR234 may be different from that in R.trifolii. This subject will be raised later in the discussion, so it must be pointed out that there is also the possibility that the recombinant plasmid, pRs51, may not be entirely stable in Rhizobium, or may delete the inserted fragment at a high rate, therefore preventing the plant finding a nodulation proficient cell amongst the large inoculum. It is also possible, that some intrinsic quality of the vector (pSUP106) may prevent the expression of the cloned nodulation genes. The obvious way to test these possibilities is to re-clone the fragment on another

vector capable of replication in Rhizobium. At the time of these experiments no suitable vector existed, which was derived from another replicon apart from RSF1010, had suitable cloning sites and had an antibiotic resistance marker which could work in NGR234 derivatives.

7.4 CONSERVATION OF EARLY NODULATION GENE FUNCTIONS

These experiments show that Hac genes involved in the nodulation of clovers can correct a Hac$^-$ mutation in a strain which normally nodulates tropical legumes. The plasmid used for these studies was constructed by P. Schofield. He cloned a 14kb HindIII fragment from the Nod locus of the R.trifolii strain SU843 onto a vector (pKT240) derived from the IncQ plasmid, RSF1010. The recombinant plasmid, pRt032, carries all Sym plasmid-encoded Nod genes from this R.trifolii strain (Schofield et al., 1983b).

When this plasmid was transferred to strain ANU240 the resulting transconjugant was able to form small nodules on white clover, in much the same way as strain NGR234 (pBR1AN), described in Chapter 4. pRt032 was transferred to the Sp^R derivative of mutant 16-5 (ANU1256). The strain ANU1256 (pRt032) formed micronodules and caused root beading on clover in the way of ANU240 (pRt032). As a control pRt032 was transferred to strain ANU265, where it gave a similar phenotype on

clover as ANU1256 (pRtO32). Strain ANU265 (pRtO32) did not nodulate siratro.

Strain ANU1256 (pRtO32) was able to form nodules on siratro, but at a reduced frequency in comparison to the wild-type control strain ANU240 or strain ANU240 (pRtO32). The corrected mutant gave about one to two nodules per plant, compared with the normal number of five to ten. Bacteria reisolated from nodules had no improvement in nodulation frequency with siratro and were still able to form small nodules on white clover. M. Djordjevic continued these studies, showing that cloned R.meliloti Hac genes on the plasmid pRmSL26 (Long et al., 1982) and the R.leguminosarum Nod genes on pRL1JI (the parent of pJB5JI) were unable to complement the 16-5 mutant for nodulation of siratro.

A curious observation in this work was the complete lack of expression of pRmSL26 Hac genes in strain ANU240 and ANU265. In other words strains ANU240 (pRmSL26) and ANU265 (pRmSL26) were unable to induce root hair curling on lucerne. This is a contrast to the result of Kondorosi et al. (1982) who showed that the entire R.meliloti megaplasmid when in strain NGR234 could induce small nodules on white clover. To confirm this observation the plasmid (pRme41b:pAK11) was received from A. Kondorosi and transferred to the heat-cured strain, ANU265. The transconjugant strain ANU265 (pRme41b:pAK11) was able to form small callus-like nodules on lucerne; essentially the

same result as reported by Wong *et al*. (1983) and Kondorosi *et al*. (1982) for the same plasmid in NGR234. This result shows that the entire *R.meliloti* Sym plasmid can give nodulation of lucerne in ANU265 while the smaller clone of the *R.meliloti* Hac region (pRmSL26) can not. This agrees with Kondorosi *et al*. (1983) who described two separate loci essential for nodulation in *R.meliloti*. The plasmid pRmSL26 is thought to contain only one of the required Nod gene loci in *R.meliloti*.

7.5 CROSS-HYBRIDISATION TO *RHIZOBIUM TRIFOLII* HAC GENES

Since functional complementation could occur between a Hac^- Nod^- mutant of the fast growing cowpea *Rhizobium* strain and cloned nodulation genes of *R.trifolii*, it infers that some kind of allelic form of the same gene exists in both DNA regions. This section describes attempts to identify such genes by using DNA hybridisation.

The entire 14kb *Hin*dIII fragment which contains all known *R.trifolii* hair curling and nodulation genes (Schofield *et al*., 1983b) was used as a probe against total genomic DNA of ANU240. Large amounts of ANU240 DNA (about 20 *u*g) were digested for this analysis since previous attempts to show cross-hybridisation failed. The probe DNA (1 *u*g, a gift from P. Schofield) was radioactively labelled to high activity using nick

translation with both radioactive dATP and dCTP. About 2 x 10^6 dpm of incorporated label was allowed to hybridise for two days at 60°C, to allow maximum binding to homologous DNA sequences. The filter was washed four times in 2 x SSC for 15 minutes each time and then washed in 1 x SSC at 65°C for two hours. Then the filter was exposed to sensitive X-ray film (Kodak XAR-5) for 7 days with intensifying screens. The result is shown in Figure 7.7. The hybridisation is extremely weak given the excessive conditions favouring binding of the probe to the filter. A perfectly homologous probe of similar size hybridised under these conditions only needed several hours exposure to register a comparable image on the X-ray film. It can therefore be concluded that the conservation of DNA sequence homology is not extensive or that the region of conserved homology is very small.

Despite the weak hybridisation, the R.trifolii Nod gene probe faithfully identified the Hac region defined by the mutant 16-5. Fragments characteristic of the ANU240 Nod region are obvious in the hybridisation shown in Fig. 7.7. An important observation is the fact that the intensity of hybridisation to a 5.5kb SalI fragment is about the same as to the 16kb XhoI fragment. This result suggests that the majority of significant hybridisation is confined to this SalI fragment, which maps within the 6.7kb HindIII fragment. A diagrammatic representation of the likely zones of homology between the R.trifolii "Nod"

region and NGR234 DNA is given in Figure 7.8.

A small PstI fragment of 700bp which entirely contains the gene identified by the R.trifolii Hac$^-$ mutant ANU851 (Schofield et al., 1983a) was also used as a probe. This fragment was cloned in the bacteriophage M13-mp10 (gift of P. Schofield) and single stranded DNA used to prepare a radioactive probe by using a sequencing primer, DNA polymerase I (large Klenow fragment) and radioactively labelled dCTP, and unlabelled dATP, dGTP and dTTP. The probe synthesized in this manner contained about 2.5×10^6 dpm.

The probe was hybridised to a blot of multiple digests of pure pRs1 and pRs5 DNA. Since pRs1 contains both the 6.7kb and 2.3kb adjacent HindIII fragments and pRs5 contains the adjacent 9.4kb HindIII piece, the amount of DNA from the Nod region analysed for hybridisation is 18.5kb of contiguous DNA. The hybridisation was done at 55^{o}C for three days, followed by stringent washing conditions. The filters were exposed overnight to X-ray film to obtain the image shown in Figure 7.9. The probe hybridised to fragments of pRs1 but not pRs5. In pRs1 the hybridisation was confined to the 2.8kb EcoRI fragment (the same fragment in which Tn5 is inserted in the mutant 16-5) and to the 1.3kb BamHI and 2.6kb BamHI-HindIII fragments. As the probe is such a small piece of DNA it is likely that the zone of homology between pRs1 and the R.trifolii Hac gene probe is confined to a small zone of a

few hundred base pairs long which spans the BamHI site as shown in Figure 7.8. This map position is within a few hundred base pairs of the site of insertion of Tn5 in the mutant 16-5.

A sub-clone of the 14kb HindIII fragment, a 2.05kb EcoRI-BamHI piece, was used as a probe against total genomic ANU240 DNA, under the same conditions as for the 14kb HindIII fragment. This fragment hybridised to the ANU240 fragments as shown in Figure 7.8.

The hybridisation comparison was further extended with the entire 16kb XhoI fragment was cloned. Multiple enzyme digests of this fragment and DNA from lambda 3 and lambda 5 were probed with two sub-clones of the 14kb HindIII piece from R.trifolii. These were the two end HindIII-EcoRI fragments: pRt590, a 4kb HindIII-EcoRI fragment; and pRt011, a 3kb EcoRI to HindIII fragment. Neither of these fragments hybridised significantly to the 24kb of DNA represented by the lambda clones and pRs50. Even after a 7 day exposure with intensifying screens no "Nod" fragments showed hybridisation. This result is consistent with all homology between NGR234 and the R.trifolii "Nod" fragment being contained entirely within the small internal region of both DNA where Hac^- mutants are known to reside. Less stringent hybridisation conditions may be needed to detect further zones of weak homology between the two "Hac" regions. Rather than pursue such experiments, it was thought that DNA

sequencing would be a better strategy to analyse the extent of divergence of these two Hac gene loci.

7.8 DISCUSSION

The gene mutated in 16-5 is obviously essential for nodulation on a range of plants, including Parasponia. The DNA region around this region is capable of hybridising with Hac genes from R.trifolii suggesting the existence of allelic forms of these genes in NGR234. This observation was verified by the biological complementation of the 16-5 mutant with the 14kb R.trifolii Nod region. Just how this complementation can occur is not understood but it may be that an R.trifolii Hac gene product can cross react enough with siratro root hairs to give the low level nodulation of siratro.

The Southern analysis in the circuit experiment suggested that the 6.7kb HindIII fragment contains an entire transcriptional unit. It is not known whether this unit is a single gene or an operon comprised of several genes. Given that all the hybridisation between the R.trifolii Nod region was contained in the 5.5kb SalI fragment which is within the 6.7kb HindIII fragment, and that a 5.5kb BglII fragment from the R.trifolii Nod region contains a functional Hac gene region (M. Djordjevic, personal communication) it was surprising that the 6.7kb HindIII could not permit the expression of the Hac

phenotype in ANU265. It is even more surprising that the 16kb XhoI fragment likewise was unable to give a Hac^+ phenotype. There are three possible explanations. The first is that the 16kb XhoI fragment contains all the genes required for root hair curling and nodulation, but needs another, unlinked, function for positive activation of these genes. The second is that the 16kb fragment contains some genes for root hair curling and/or nodulation but another locus contains other genes essential for root hair curling and/or nodulation. The third possibility is a subset of the second; it is possible that one of the XhoI sites is by chance in the middle of a gene essential for nodulation, and is therefore non-functional on pRs51. All of these possibilities lead to the conclusion that genes responsible for nodulation are not linked in a transcriptional sense in NGR234.

The location of the other nodulation gene locus on pRspNGR234a is unknown. At the end of this study Dr. M. Nayudu was able to isolate R-prime plasmids using the U7 mutant (recall that U7 is Fix^- and has Tn5 in the Sym plasmid). R-prime plasmids are large recombinant plasmids constructed *in vivo*, and may contain many hundreds of kb of DNA. A comparison of R-primes which can confer a Nod+ phenotype in ANU265 to those which are Nod^- in ANU265 should allow identification of the other Nod gene locus. Alternatively, if the third case above is correct it may

be possible to clone all the the Nod genes by isolating some larger restriction fragments. These problems will have to be overcome before an understanding of the control of host range at the early infection stage is possible.

The conservation of DNA homology is not great between R.trifolii Hac genes and the cloned DNA fragments from the 16-5 Hac locus. Only DNA sequencing can provide a full analysis of the differences between these two Hac gene loci. Whatever constraints have existed to conserve DNA homology at the Hac gene region they are certainly not as strong as the constraints on nitrogenase enzyme function which have kept the strong homology between the nifH and nifD genes of nitrogen fixing organisms (Ruvkun and Ausubel, 1980; Scott et al., 1983a,b). The identification of nif genes in Chapter Four did not require the extreme conditions needed to demonstrate cross-hybridisation between the Hac genes.

The only example so far where two separate loci are required for nodulation has come from the laboratory of Dr. A. Kondorosi. Kondorosi et al. (1983) described a region of about 8kb (cloned on plasmid pKSK5) from the R.meliloti strain 41, which codes for root hair curling. This region is essentially the same as that described by Long et al. (1982) which is in the plasmid pRmSL26. The hair curling gene region of pKSK5 can be replaced by the Hac regions of R.trifolii and R.meliloti and is the region deleted in the mutant ZB157 (recall that ZB157 is a

deletion mutant of R.meliloti and can be complemented for lucerne nodulation by pBR1AN, pJB5JI but not pNM4AN). About 12kb away from the Hac region in R.meliloti strain 41 is another locus essential for nodulation. Deletions which remove the first and the second Nod gene loci cannot be complemented by pBR1AN, pJB5JI or pKSK5. Kondorosi et al. (1983) suggest that this is a gene region which is involved in the host specific control of nodulation. The second Nod locus (designated Hsp) has been mapped by Tn5 mutagenesis and appears to contain two small genes of about 1kb each (A. Kondorosi, personal communication). Just how these genes interact with the Hac genes or the plant is not known. Certainly this arrangement of Nod genes is entirely different from those in R.leguminosarum (Downie et al., 1983a) and R.trifolii (Schofield et al., 1983b) where Hac genes are intimately linked with genes governing nodule induction and host specificity. At present nothing has appeared in the literature concerning the arrangement of nodulation genes in slow-growing rhizobia of any sort. Although genes from a slow-growing Parasponia Rhizobium were reportedly cloned by complementation of a Tn5 induced Hac$^-$ R.meliloti mutant (Marvel et al., 1984) nothing was reported about the organisation of the genes.

It is worth noting again the anomalous behaviour of pNM4AN in ZB157. When pBR1AN and pJB5JI were transferred to ZB157 they both could complement lucerne nodulation but

could not give nodulation of clovers or peas, respectively. It seems that some gene in ZB157 was preventing the normal expression of host range function on these Sym plasmids. The situation with pNM4AN in ZB157 is completely the reverse; no complementation of lucerne nodulation occurs and the strain is effective in symbiosis with siratro. Whatever prevents expression of Nod functions on pJB5JI and pBR1AN obviously has little effect on pNM4AN. This implies that the control of expression of Hac and Nod genes on pNM4AN is different from that on pJB5JI, pBR1AN or in R.meliloti.

SUMMARY

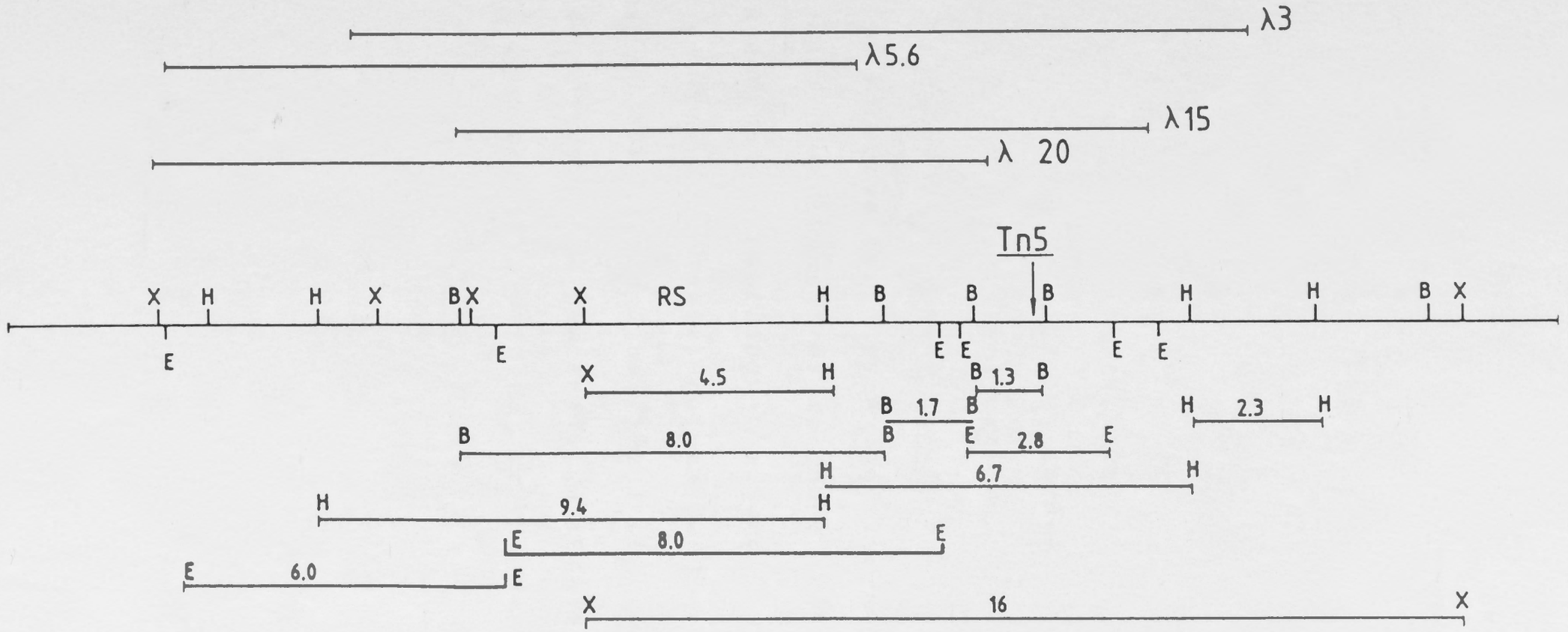
1. The Hac$^-$Nod$^-$ mutant 16-5 cannot nodulate any tested plant and is apparently blocked for root hair invasion as well as the mode of infection used in Parasponia nodulation.

2. The DNA region around the 16-5 mutation on the Sym plasmid has zones of homology with the R.trifolii Nod genes. This homology was verified by the biological complementation of 16-5 by R.trifolii Nod genes, for siratro nodulation.

3. A 6.7kb HindIII fragment, which contained all homology with known Hac genes in R.trifolii, and which contained at least one transcriptional unit, was unable to confer a Hac$^+$ phenotype in a heat-cured strain.

4. A large fragment (16kb XhoI) which spans the site of insertion in 16-5, was unable to confer a Hac$^+$ or Nod$^+$ phenotype when introduced to the heat-cured strain on the vector, pSUP106.

Figure 7.1. Map of the DNA region cloned, flanking the site of Tn*5* insertion in the Hac$^-$ mutant 16-5. The insert DNA contained in lambda recombinants is shown above, whilst the DNA cloned in various plasmids is shown below. The site of insertion of Tn*5* is indicated. The approximate position of a repeated DNA region in the DNA is shown by the RS.



10 KILOBASES

Figure 7.2. Example of the method used to verify the restriction map of lambda recombinant phages. Lambda 20 is a real phage with an insert size of about 18kb, which originates in a 2.8kb EcoRI fragment as shown in Figure 7.1. This phage also has a repeated sequence which is present in at least 5 copies in the genome. Lambda 16 is an example of a "monster" recombinant which has a small portion from the "Nod" region (about 4kb) and the rest of the insert DNA is not derived from the ANU240 genome. The large XhoI fragment, often mentioned in the text, is seen in both these blots. Letters represent: C, ClaI; H, HindIII; X, XhoI; E, EcoRI; S, SalI.

λ-20

kb C H X E S

23-
9·4-
6·5-
4·3-
2·3-
2·0-
0·5-

λ-16

kb C H X E S

23-
9·4-
6·5-
4·3-
2·3-
2·0-
0·5-

Figure 7.3. Hybridisation using the 6.7kb HindIII fragment against digests of total ANU240 DNA. Digests are: track 1, EcoRI; track 2, HindIII; track 3, BamHI; track 4, XhoI; track 5, SalI; track 6, PvuII; track 7, KpnI; track 8, BclI; track 9, BglII; track 10, ClaI; track 11, PstI. Exposure was 16 hours with one intensifying screen at -80°C.

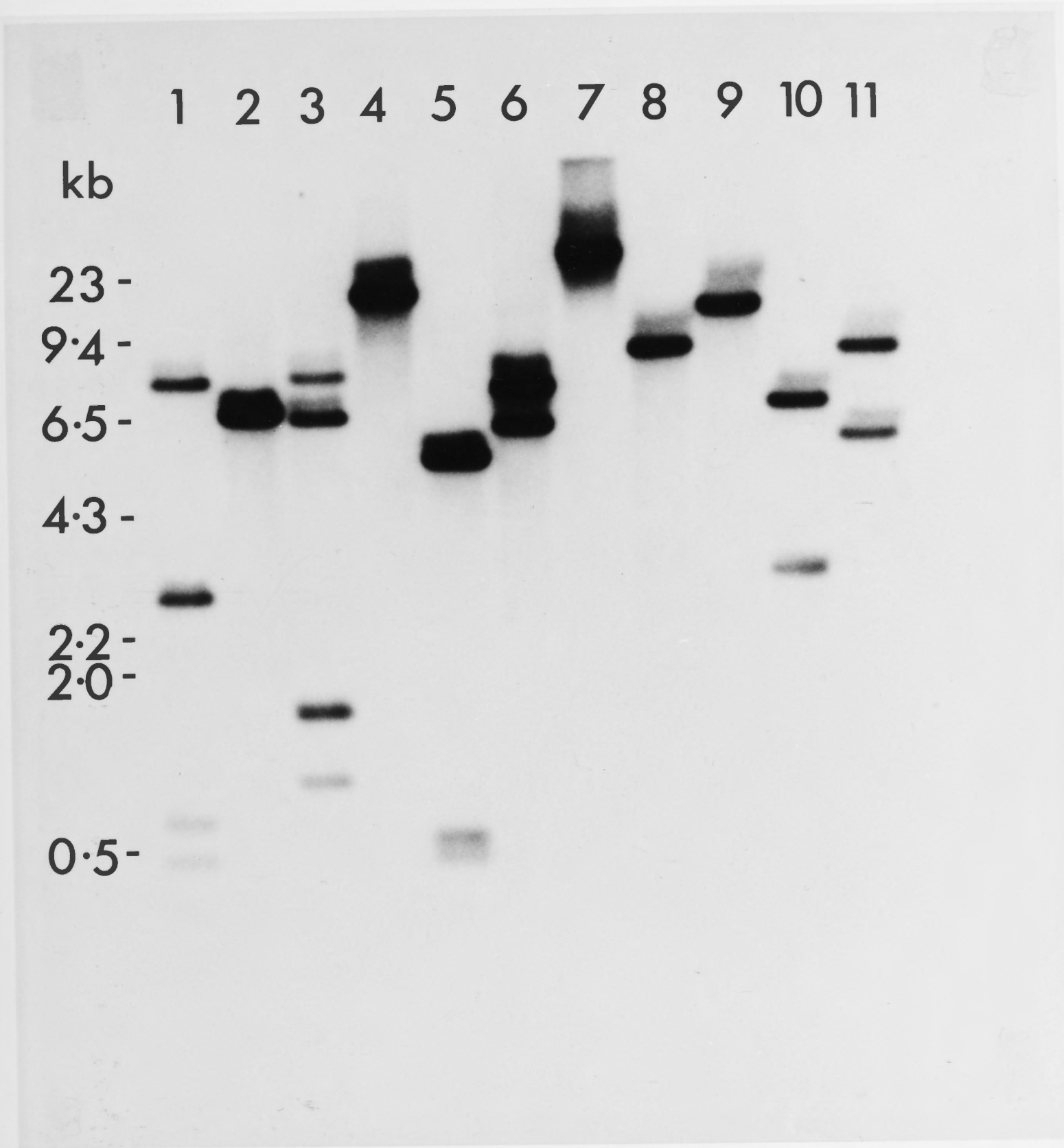

1
2
3
4
5
6
7
8
9
10
11
kb
23 -
9·4 -
6·5 -
4·3 -
2·2 -
2·0 -
0·5 -

Figure 7.4. Circuit analysis using HindIII digests of ex-nodule isolate strains. The probe used was pRs23, the pSUP202 recombinant containing the 6.7kb HindIII fragment from the NGR234 Hac region. Track 1 shows the wild-type strain ANU240 with a normal 6.7kb HindIII. Track 2 shows the mutant 16-5, which has Tn5 inserted in the 6.7kb HindIII fragment. Since there are HindIII sites 1.1kb from each end of Tn5, the 6.7kb HindIII fragment has been cut in two in the mutant 16-5. Tracks 3, 4, 6 and 7 are "corrected" Nod^+ ex-nodule isolates of ANU1265 (pRs23). These strains are Km^R, Cm^R. Recall that ANU1256 is a Sp^R mutant of 16-5. These strains have the arrangement of fragments found in 16-5, as well as a normal 6.7kb HindIII fragment. The larger fragment of about 8.5kb is pSUP202. The intensity of hybridisation between bands in the same track suggests an equal copy number. Intensity varies between tracks due to differences in the amount of DNA run on the gel. Tracks 11 and 12 are ex-nodule isolates of ANU1256 (pRs21). These strains are Km^S, Cm^S. Only the wild-type 6.7kb HindIII band is present, showing that correction has occurred by double reciprocal crossover, with subsequent loss of the pRs21.


kb
1
2
3
4
5
6
7
11
12
9·4-
6·5-
4·3-

Figure 7.5. Circuit analysis using XhoI digestion. The probe used was pRs50, a pACYC177 recombinant which contains the entire 16kb XhoI fragment. The strains used were the same as in Figure 7.4. Track 1 is strain ANU240, which shows the normal 16kb XhoI fragment, plus bands caused by the repeated DNA element which maps at one end of this fragment. Track 2 shows the 16kb XhoI fragment has been split into two fragments of 8 and 9kb in the mutant 16-5, since Tn5 has XhoI sites near its ends. Tracks 3 and 4 are examples where pRs23 has recombined on one side of the Tn5 insertion to give phenotypic correction of the Hac^- mutant 16-5. Because pRs23 has no XhoI site it has increased the size of the XhoI fragment in which it has recombined. In tracks 3 and 4 it has recombined in the 9kb fragment normally present in 16-5. In tracks 6 and 7, pRs23 has recombined on the other side of the Tn5, increasing the size of the 8kb XhoI fragment. In all these tracks the repeated sequence hybridisations are unchanged. These results show that pRs23 can recombine on either side of the Tn5 in the mutant 16-5 to give phenotypic correction. Tracks 9 and 10 show correction by double reciprocal crossover, creating a normal 16kb XhoI fragment. The strains used for tracks 9 and 10 were the same used in tracks 11 and 12 in Figure 7.4.

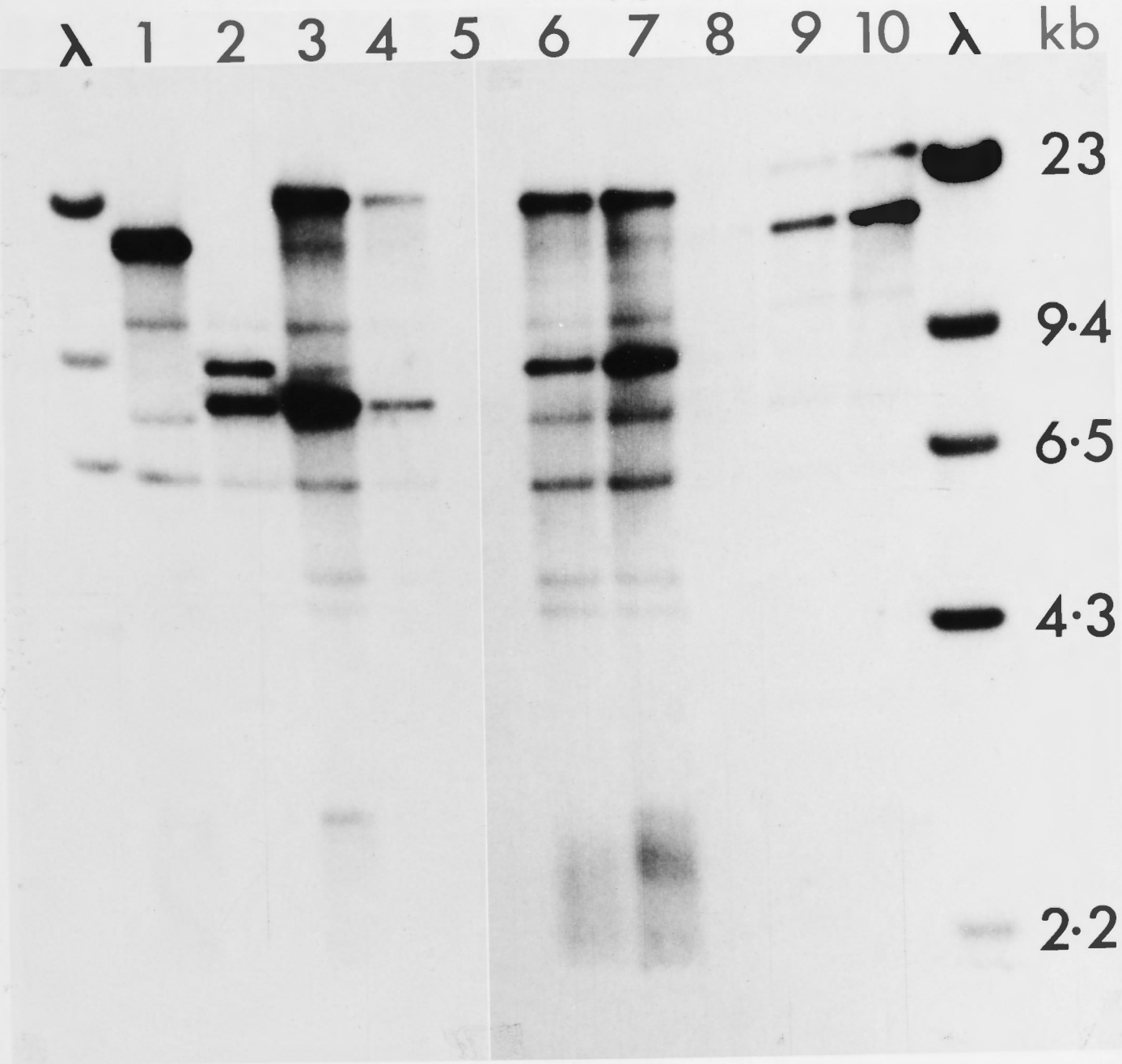

λ 1 2 3 4 5 6 7 8 9 10 λ kb
23
9·4
6·5
4·3
2·2

Figure 7.6. The possible arrangements of genes in the region defined by the 16kb XhoI fragment. Three genes (labelled a, b and c) are shown for the sake of argument, although the actual number of genes in this region is not known. The genes are displayed as transcriptional units, with P representing a hypothetical promotor sequence and the line representing the extent and direction of transcription. The first diagram shows the position of the Tn5 insert in the mutant 16-5, with the XhoI sites in Tn5 clearly marked. The position of the XhoI sites is important to the logical analysis of the results shown in Figure 7.5. In the other diagrams the Tn5 is replaced by the arrow. The possible arrangements of genes are given in A, B, C, D and E. The sections F and G show the structure of the DNA in the corrected ex-nodule ANU1256 (pRs23) strains analysed in Figures 7.4 and 7.5. The pRs23 molecule is not shown fully, but the dashed lines should be conected. From the structures shown in F and G, it is obvious that for both these structures to give full expression of the gene mutated in 16-5 then both the promotor sequence and the end of the operon (or gene) must be on the cloned fragment (this arises from the fact that Tn5 has a polar effect on genes in the same operon, "downstream" from the promotor). These conditions are only met in D and E. D shows an operon structure for the Hac genes, while E shows them as separate transcriptional units. The direction of transcription could be either from the left or the right.

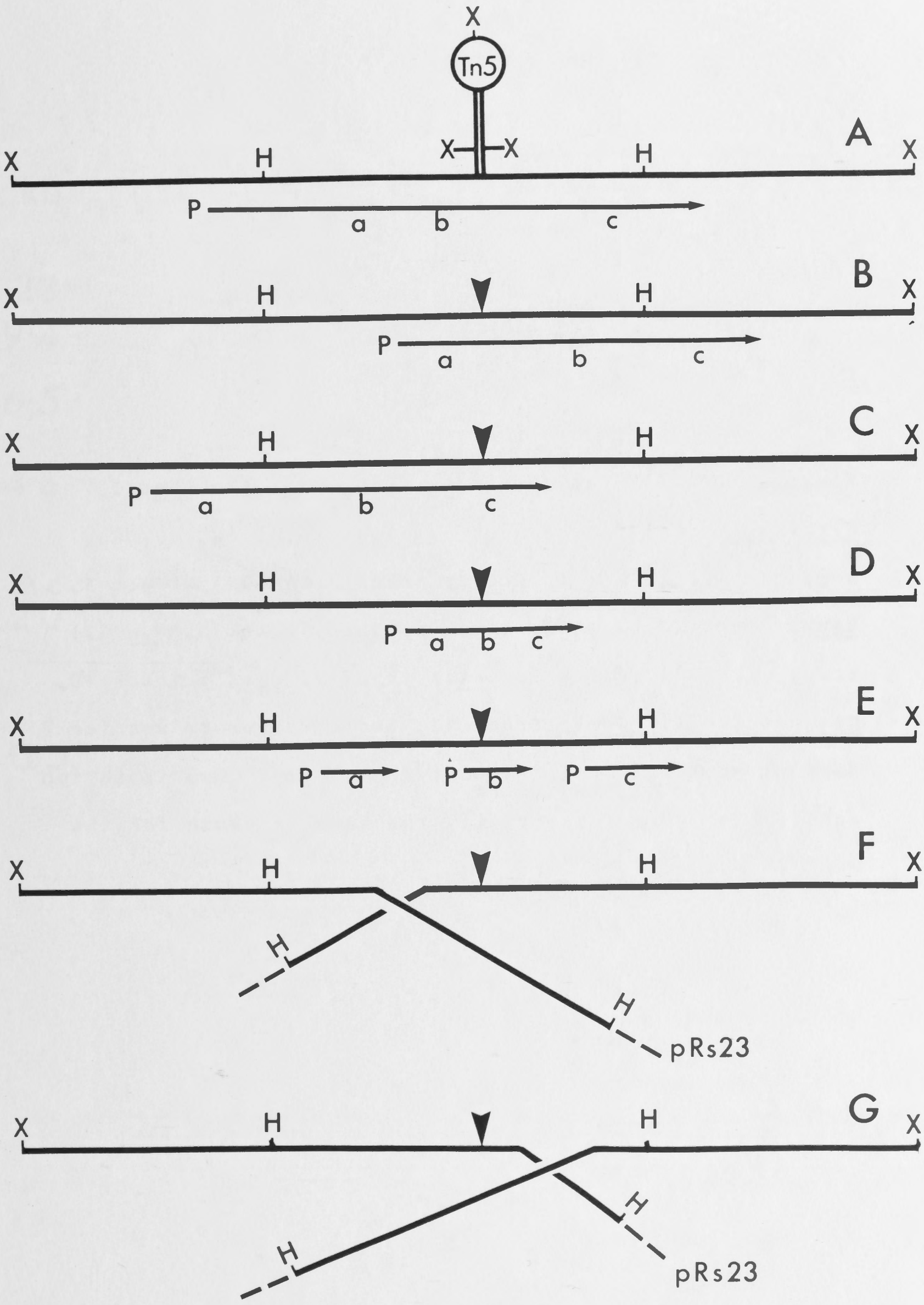

X
Tn5
X
X
A
X
H
H
X
P
a
b
c
B
X
H
H
X
P
a
b
c
C
X
H
H
X
P
a
b
c
D
X
H
H
X
P
a
b
c
E
X
H
H
X
P
a
P
b
P
c
F
X
H
H
X
H
H
pRs23
G
X
H
H
X
H
H
pRs23

Figure 7.7. Hybridisation using the 14kb HindIII *R.trifolii* "Nod" fragment against total ANU240 DNA. Digests are: track 1, EcoRI; track 2, No DNA; track 3, BamHI; track 4, XhoI; track 5, SalI; track 6, Pvu II; track 7, KpnI; track 8, BclI; track 9, BglII; track 10, ClaI; track 11, PstI; track 12, SmaI. Exposure was for 7 days at -80°C with an intensifying screen. Hybridisation conditions were essentially the same as those for the blot in Figure 7.3

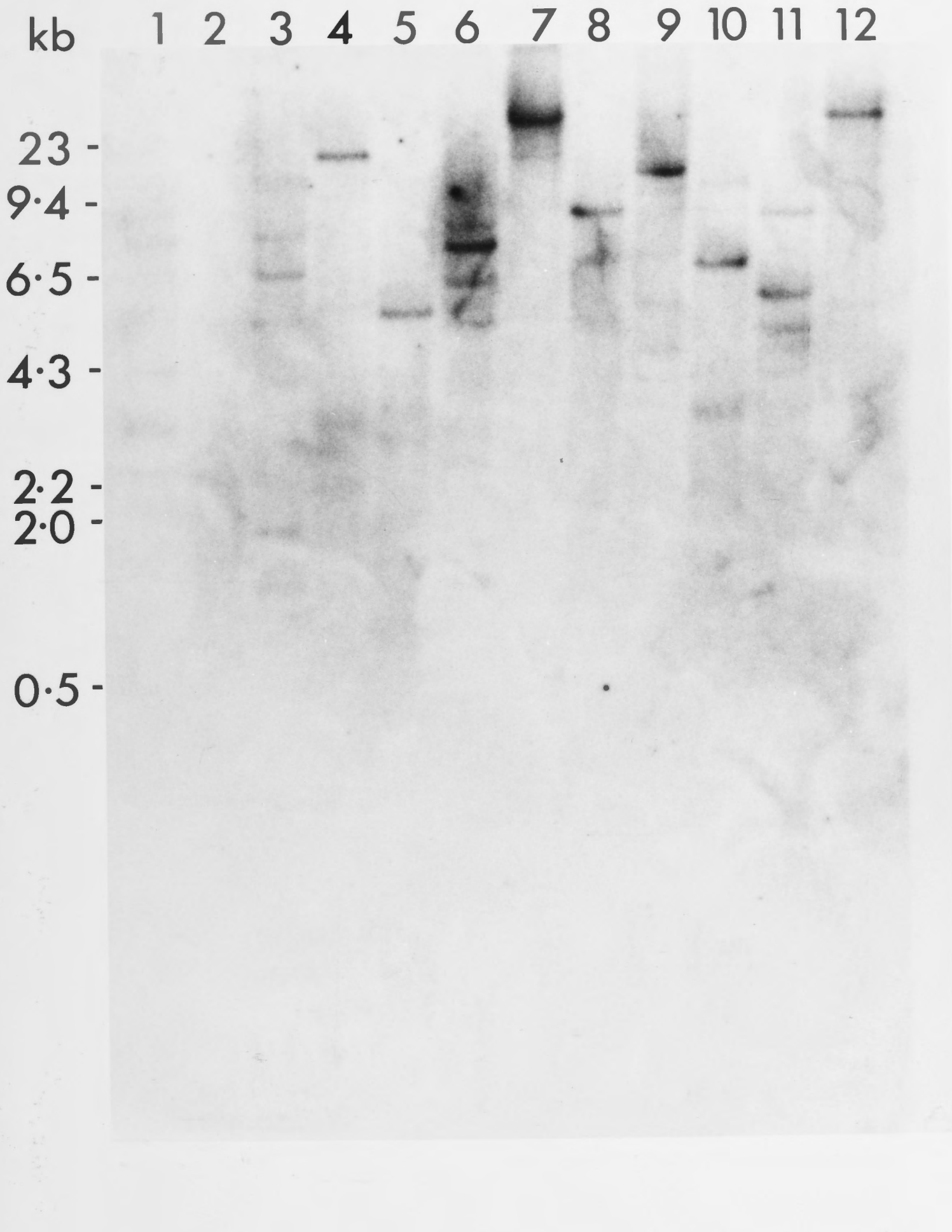

kb
1 2 3 4 5 6 7 8 9 10 11 12
23 -
9·4 -
6·5 -
4·3 -
2·2 -
2·0 -
0·5 -

Figure 7.8. Diagrammatic representation of the extent of homology, detectable by DNA hybridisation, between the NGR234 Hac region and the R.trifolii Nod region. The R.trifolii fragment is shown with confirmed Hac$^-$ mutations induced by Tn5. The BglII fragment shown in the R.trifolii map is believed to contain all the genes required for clover root hair curling, while genes responsible for other early nodulation events are thought to map close by in adjacent DNA. Mutations in the right end EcoRI-HindIII piece (referred to in the text as pRt011) have no effect on the nodulation phenotype of the R.trifolii Nod fragment. The PstI fragment shown is referred to in the text. This fragment was used in the hybridisation shown in Figure 7.9. The 2.05 kb EcoRI-BamHI fragment (pRt295) hybridises near the site of insertion of Tn5 in 16-5. This is the region most likely to contain genes allelic to the "16-5" gene. The "zone of strongest homology" corresponds to the approximate extent of detectable homology. Other regions of weak homology may exist, but have not been detected. In this diagram it is easily seen that the 16kb XhoI fragment from NGR234 is large enough to accomodate a core of Hac genes and whatever ancillary genes are required for nodulation. If the arrangement of nodulation genes in NGR234 was the same as, or similar to, that in R.trifolii, then the XhoI fragment would certainly be capable of conferring a Nod$^+$ phenotype to a heat-cured strain.

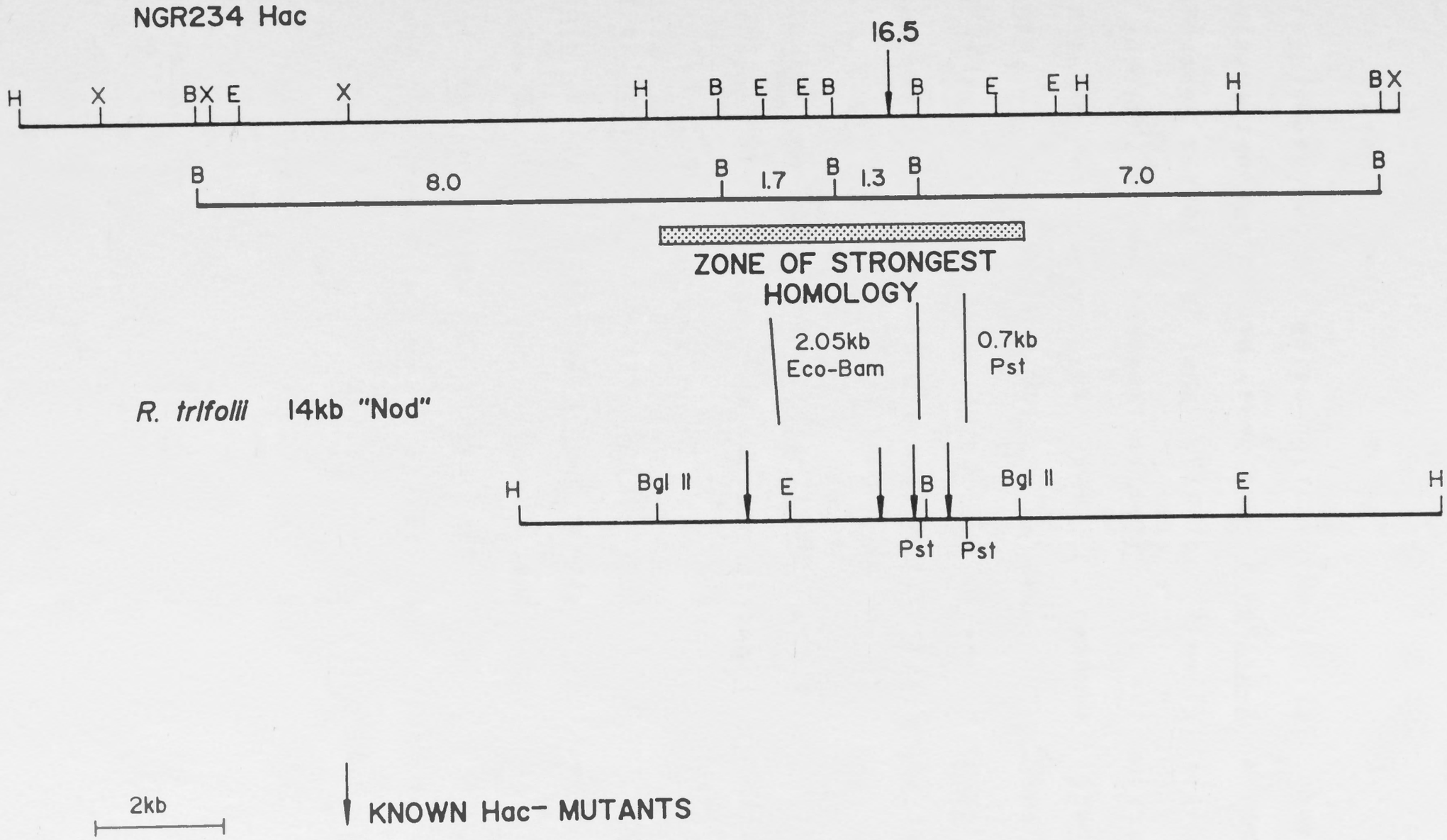
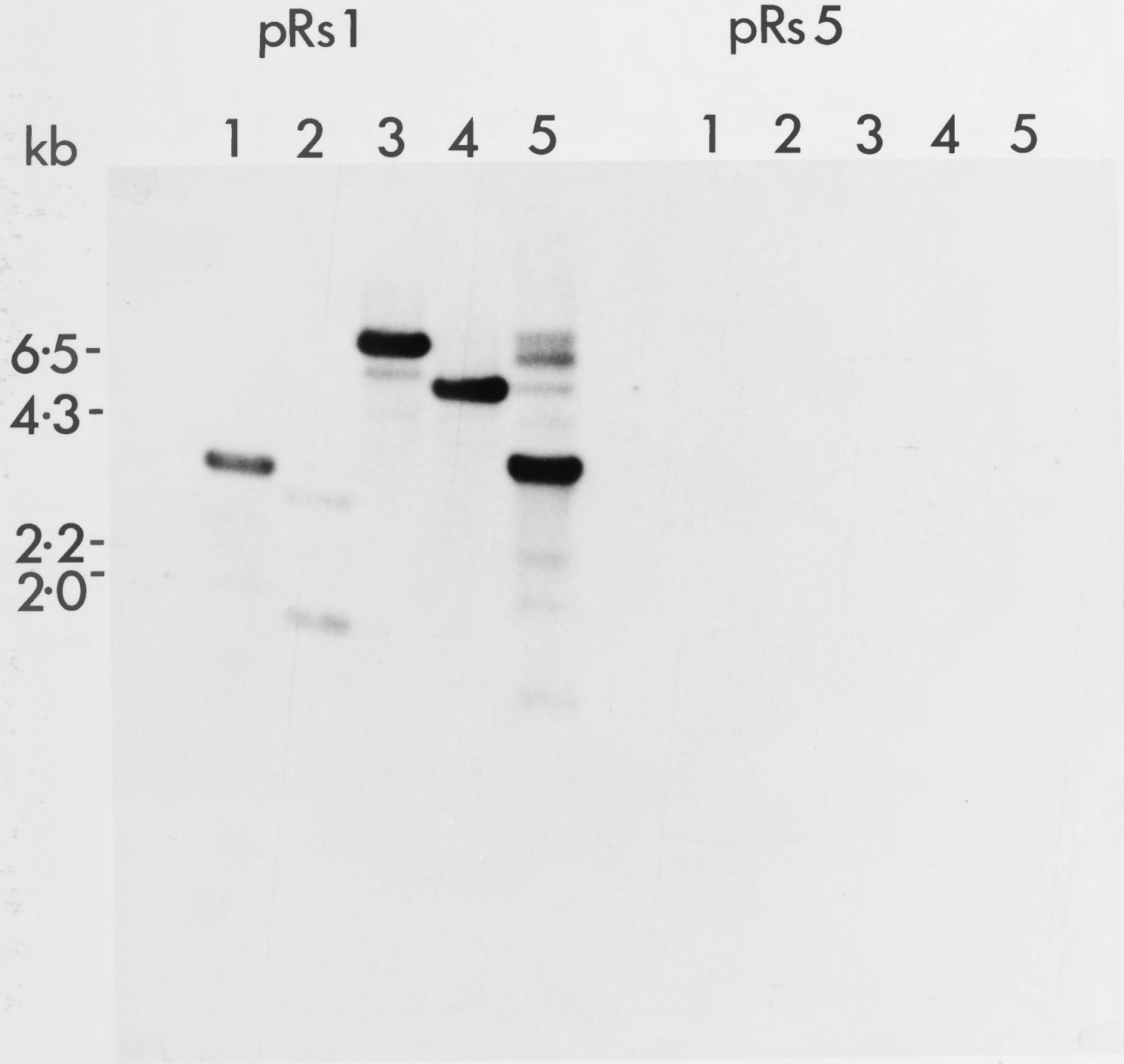

NGR234 Hac
16.5
H X BX E X H B E E B B E E H H BX
B 8.0 B 1.7 B 1.3 B 7.0 B
ZONE OF STRONGEST HOMOLOGY
2.05kb Eco-Bam
0.7kb Pst
R. trifolii 14kb "Nod"
H Bgl II E B Bgl II E H
Pst Pst
2kb
KNOWN Hac– MUTANTS

Figure 7.9. Hybridisation using a 700bp probe derived from a R.trifolii Hac gene, probed against multiple digests of pRs1 and pRs5. pRs1 is a pBR322 recombinant carrying the 6.7kb HindIII fragment and an adjacent 2.3kb HindIII fragment. pRS5 is a recombinant of pNAM2 (Appendix 1) carrying a 9.4kb HindIII fragment which is adjacent to the 6.7kb HindIII fragment. Present in this blot is 18.5kb of contiguous DNA derived from the NGR234 Hac region.

pRs1: track 1, HindIII-EcoRI; track 2, BamHI-HindIII; track 3, HindIII; track 4, SalI-HindIII; track 5, PstI-HindIII.

pRs5: track 1, HindIII-XhoI; track 2, HindIII-SalI; track 3, HindIII-ClaI; track 4, HindIII-PstI; track 5, HindIII.

This blot shows that the probe hybridisation is entirely within a 2.8kb EcoRI fragment (in which Tn5 maps in the mutant 16-5), and overlaps a BamHI fragment of 1.3kb and a HindIII-BamHI piece of 2.6kb.


pRs1
pRs5
kb
1 2 3 4 5
1 2 3 4 5
6·5-
4·3-
2·2-
2·0-

CHAPTER EIGHT

GENERAL DISCUSSION

The strain NGR234 has proven to be an excellent organism for studies into the genetic basis for nodulation of legumes and non-legumes, and the differences in arrangement of symbiotic information between fast- and slow-growing rhizobia of the cowpea group. This strain provides the opportunity to investigate the symbiotic process in plants normally nodulated by slow-growing Rhizobium strains. It also permits studies on the differences between various host plants, eventually to unravel the remarkable broad symbiotic ability of NGR234.

When work began on the genetics of NGR234 in 1981, Sym plasmids had just been identified as a general phenomenon in classical fast-growing Rhizobium strains, and transposon mutagenesis was gaining acceptance as a novel method for the isolation of symbiotic mutants. Molecular cloning of the actual genes involved in symbiosis had been achieved for the nifHDK operon in R.meliloti (Ruvkun et al., 1982) and for at least the nifH gene of R.japonicum (Hennecke, 1981). However, genes for early symbiotic traits such as root hair curling and infection thread formation remained elusive, although several laboratories had presumptive Tn5 induced mutations

in early nodulation genes. The interspecific transfer of Sym plasmids was being investigated in *R.leguminosarum*, *R.trifolii*, and *R.phaseoli*, but Sym plasmids had not been identified in more exotic strains of *Rhizobium*.

The work presented in this thesis provides the basis for continued work into the symbiotic ability of strain NGR234. Tn*5* mutagenesis coupled with molecular cloning will conceivably allow the isolation of almost any gene involved in symbiosis between NGR234 and the large number of plants capable of being infected by NGR234. The mobilisable Sym plasmid, pNM4AN, will be useful in future studies on interactions between Sym plasmids. Interactions between Sym plasmids and the bacterial chromosome is another aspect of *Rhizobium* research which will expand in the future. This sort of research will give insight into the characters required to infect certain plants and not others. For instance, the data already acquired from various laboratories (Table 5.1) indicates that *R.meliloti* behaves differently to *R.trifolii*, *R.leguminosarum* and NGR234 when foreign Sym plasmids are introduced. Further biochemical characterisation of the genes, and their products, involved in the process of symbiosis will enable studies of their interaction with the plant host and the bacterium, to answer whether the symbiotic partners communicate chemically during nodule development.

The *nif* genes of NGR234 are arranged quite

differently from those in slow-growing cowpea or soybean rhizobia. The nifH gene is located on a separate operon from the nifDK operon in the slow-growing cowpea strain CP283 (Scott et al., 1983b), and the slow-growing R.japonicum strain USDA110 (Fuhrmann et al., 1984). In contrast, the nif gene arrangement in NGR234 is very similar to that in R.phaseoli (Quinto et al., 1982), where duplicate nifHDK operons exist on the same plasmid (Badenoch-Jones et al., 1984). These results indicate that the arrangement of the nif gene cluster has little to do with the broad-host-range effectiveness character of cowpea Rhizobium strains, both fast and slow. Further work has demonstrated a closer sequence homology between the nifH gene of NGR234 and other fast-growing Rhizobium strains rather than to the nifH gene of slow-growing strains (J. Badenoch-Jones, personal communication). Present work with NGR234 nif genes involves creating insertional mutations in each of the duplicate nif genes to investigate whether both nif operons are functional. Other work should be directed at analysing the Fix^- mutant U7 to see if it also resides in a duplicated DNA region. It will be interesting to see if fast-growing R.japonicum strains have an arrangement of genes similar to that of NGR234, or conceivably have an entirely different arrangement.

It is obvious that NGR234 is only superficially similar to slow-growing cowpea strains. The symbiotic

host range is perhaps the only trait in common. NGR234 is very similar to fast-growing R.japonicum strains and fast-growing rhizobia from temperate legumes in the arrangement of symbiotic gene information on large Sym plasmids. It is apparent that there is no obvious reason why slow-growing Rhizobium dominate the cowpea group of plants, since even R.meliloti carrying the correct plasmid can nodulate siratro.

The compatibility displayed by the NGR234 Sym plasmid (pNM4AN, Chapter Five) for fast-growing rhizobia and its complete inability to become established in slow-growing strains argues that the Sym plasmid is native to a fast-growing strain. It was a possibility that the Sym plasmid was derived from a slow-growing cowpea strain of some kind and transferred to a fast-growing strain in some type of conjugation event. The complete lack of Sym plasmids (so far) in slow-growing strains also argues that the NGR234 Sym plasmid has evolved in a fast-growing strain. Conceivably, NGR234 could have been created by some type of recombination or DNA transfer between a Leucaena Rhizobium and a slow-growing cowpea strain, such that the host-range determinants, and perhaps nodulation genes, were inserted into the "proto" NGR234. Alternatively, the nodulation genes of such a "proto" NGR234 could have undergone "micro-evolution" at the DNA level to acquire the broad infection trait. Only futher work on the DNA sequence of nodulation gene regions of a number of

Rhizobium strains can answer this question. These types of theories may explain the evolution of an infective host-range as broad as that of NGR234, but further changes must occur to create an organism with a broad effective ability (since plant and bacterially determined factors affect the effective host range, as shown in Chapter Three). Chromosomally determined factors obviously exist which affect the ability of a particular *Rhizobium* strain to establish an effective symbiosis with a particular plant. Further work is needed to establish how this phenomenon occurs.

The virulence of plant-pathogenic bacteria is dependent upon exo- and lipopolysaccharides (reviewed by Vance, 1983). Avirulent mutant bacteria which had altered exopolysaccharide or lipopolysaccharides are likely to set off the host plants' protective hypersensitive response, where plant cells die in the infected zone in an effort to contain the infection. It is tempting to speculate on similar models for the behaviour of the *Rhizobium*-plant interaction, especially *Rhizobium* mutants that are altered in exopolysaccharide. Recent work with NGR234 has shown that changes in the exopolysaccharide phenotype leads to an alteration in the effective host range (H. Chen, personal communication). Tn*5*-induced mutant strains have been isolated which are effective nodulators of siratro but only produce a defective, callus-like nodule on *Leucaena leucocephala*. The reverse phenotype was also

possible. This type of behaviour is very similar to that observed in this study for Rhizobium strains carrying exogenous Sym plasmids (Chapter Four and Five). In R.trifolii, as well, changes in exopolysaccharide have a deleterious affect on the expression of late symbiotic functions. Chakravorty et al. (1982) studied a mutant, ANU437, which was totally devoid of polysaccharide. This strain made small, ineffective nodules on clover. These nodules did not develop nitrogen-fixing bacteroids and senesced early. The exact biochemical reason for this phenomenon is obscure.

Exopolysaccharide or lipopolysaccharide differences between strains may explain differences in symbiotic ability on various hosts, if a change in the external capsule alters the ability of a Rhizobium cell to suppress or avoid setting of the plant defence system. In this scenario, the bacterium would be able to initiate infection but during infection the plant would respond, recognising the Rhizobium as a foreign entity and triggering an appropriate response. Whether this response is active, as in the elaboration of inhibitory compounds, or passive, as in cell death associated with hypersensitivity, the result would be the same; an inability to develop a normal effective nodule. These hypotheses can be tested in the future by comparing plants infected with pathogenic organisms to plants infected with various Rhizobium-Sym plasmid combinations.

In Chapters Four and Five it was implied that non-Sym plasmid encoded functions influence the host range of effective symbiosis. It is now possible to investigate whether these functions are involved with exopolysaccharide phenotypes, by transferring the NGR234 Sym plasmid (pNM4AN) into a range of exopolysaccharide mutants of various strains. The reverse experiments are also possible since a large number of such mutants are now available in NGR234 (H. Chen, personal communication) and R-prime plasmids have been constructed which contain genes for exopolysaccharide synthesis.

In Chapter Seven it was seen that the arrangement of early nodulation (Hac) genes on the Sym plasmid of NGR234 is certainly different to the situation in R.trifolii and R.leguminosarum where Hac and presumed Nod genes are linked on fairly small DNA pieces, 14kb for R.trifolii (Schofield et al., 1983b) and 10kb for R.leguminosarum (Downie et al., 1983a). In NGR234 even a 16kb fragment, which had at least one Hac gene centrally positioned, could not confer either Hac^+ or Nod^+ phenotypes on a heat-cured strain. It is possible that only one Hac gene (the "16-5" gene) resides in this area, and that other genes required for Hac and Nod are dispersed, or clustered elsewhere on the Sym plasmid. This problem is presently being addressed by saturation mutagenesis of the fragments cloned so far.

It is important to realise that the other Hac or Nod

gene loci in NGR234 have no detectable homology to the Nod gene region of R.trifolii. Even the homology that is present is weak, suggesting that the genes are significantly diverged in DNA sequence and, presumably, evolution. It is likely that the area of homology between the "16-5" locus and the R.trifolii Nod gene fragment is restricted to a very small DNA region. DNA sequencing, which is presently in progress, should answer this question.

It is obviously possible to have some kind of convergent evolution which leads to two extremely dissimilar organisms that have near identical host-ranges. The fast-growing strain NGR234 and the slow-growing strain CP283 are good examples. However, evidence exists for a similar situation even within R.trifolii . Jarvis et al. (1983) showed that R.trifolii strains from different Trifolium species had a varying capacity to hybridise in Southern analysis to a cloned R.trifolii Nod region. These workers used a cloned R.trifolii Nod fragment (p8002, a 13kb fragment containing Tn5, derived from a 7kb EcoRI fragment, which originated from the oft mentioned 14kb HindIII fragment) as a hybridisation probe to investigate the degree of conservation of Hac gene information among various Rhizobium strains. Not surprisingly the probe hybridised to other R.trifolii strains from red and white clover, and also hybridised, less strongly, to R.leguminosarum strains. The unexpected result was a lack

of hybridisation to the DNA of two specialist strains of R.trifolii. These strains only nodulate the Asian species Trifolium lupinaster. Another surprising result was that one R.trifolii from T.polymorphum (Caucasian clover) hybridised strongly to the Nod gene probe whilst another strain from the same host species did not hybridise at all. These results are certainly worthy of further investigation since they mean that even within a species such as R.trifolii it is possible to have nodulation genes which are not detectably homologous, but which act to nodulate the same plant. These could be examples of convergent evolution, where Rhizobium strains have evolved from very different ancestors, to nodulate the same host.

Given that the R.trifolii Nod gene region is significantly diverged from the NGR234 "16-5" locus it is perhaps not surprising that the degree of complementation was poor between the 16-5 mutant and heterologous nodulation genes. Only R.trifolii nodulation genes were able to complement 16-5 for nodulation of siratro. R.leguminosarum and R.meliloti Nod genes were unable to complement the mutant (although the possibility that complementation could occur on other host plants, apart from siratro, was not investigated). Downie et al. (1983a) suggest that there is a "core" of early nodulation genes which are essentially the same for all rhizobia. These genes are imagined to code for common biochemical functions associated with nodulation on all legumes, while

ancillary genes function to control host-range. In the light of this concept it is interesting to consider the result of Marvel *et al*. (1984), who were able to complement two Hac⁻ *R.meliloti* mutants for nodulation of lucerne by a cloned fragment from a slow-growing cowpea strain capable of nodulating *Parasponia*. This result argues that even a cowpea *Rhizobium* strain has a "core" nodulation region, which is essentially the same as that in *R.meliloti*.

However, results with the NGR234 Sym plasmid do not agree with this concept. In this study it was seen that pNM4AN (carrying nodulation genes from a fast-growing cowpea *Rhizobium*) could not complement the Hac⁻ *R.meliloti* mutant ZB157 for lucerne nodulation. When pNM4AN was transferred to the same mutants used by Marvel *et al*. (1984) again no complementation occurred (Djordjevic, personal communication). It is possible that some other function on the NGR234 Sym plasmid is capable of preventing complementation of the *R.meliloti* Hac⁻ mutants. Further experiments to investigate this possibility are now feasible using the cloned fragments from the "16-5" locus of NGR234.

The results of Banfalvi *et al*. (1981) and Djordjevic *et al*. (1983) with the *R.meliloti* deletion mutant ZB157 argues that the Hac gene function is not the basis of host specific nodulation between *R.meliloti*, *R.trifolii* and *R.leguminosarum*, since the Hac genes of *R.meliloti* could

be functionally replaced by those of R.trifolii and R.leguminosarum. Similarly complementation of R.trifolii mutants was possible with R.meliloti and R.leguminosarum nodulation genes. In these strains the basis of host-bacterial recognition and specificity must exist at some later stage (perhaps infection thread formation). Since the organisation of R.trifolii and R.leguminosarum nodulation loci seems similar (Schofield et al., 1983b; Downie et al., 1983a) and there is quite good cross hybridisation between the Nod genes of these strains (Jarvis et al., 1983) it is not surprising that functional complementation can occur. It is surprising that this can occur in R.meliloti, which has a different arrangement of genes (Kondorosi et al., 1983) and reportedly represents a genetically different class of rhizobia (Graham et al., 1964; Kondorosi et al., 1980). It is even more surprising that it can occur between a slow-growing cowpea Rhizobium strain and R.meliloti. If the "core" nodulation gene concept is correct, it is likely that the Hac gene region represents the "core" with ancillary genes controlling host range. Just how such genes could interact will be the subject of fascinating future research.

Even if there is a "core" of Hac genes which are similar in most Rhizobium strains, it should be possible for evolutionary change to affect the function of these genes to create host-specific effects. This may explain the puzzling minor differences in host-range found among

strains of the same cross-inoculation group. Increasing host-specificity of a diverged Hac gene region may be reflected by the lack of complementation of the 16-5 mutant by R.leguminosarum and R.meliloti information, and poor complementation by R.trifolii information. One would predict that good complementation would be achieved by using Nod genes from fast-growing Leucaena rhizobia, fast-growing R.japonicum and perhaps slow-growing cowpea strains. This experiment is now possible since presumptive nodulation genes have been cloned from the slow-growing strain CP283 using hybridisation probes derived from the R.trifolii Nod region (Scott, personal communication). Similarly, presumptive nodulation genes have been identified in a R.japonicum strain, using the cloned fragments from NGR234 as probes (Plazinski, personal communication).

The plasmid borne nodulation genes of NGR234 appear to represent a new type of organisation of nodulation genes, distinct from that of the other fast-growing strains so far studied, but may be similar in some respects to R.meliloti. It will be interesting to see if the organisational pattern of nodulation genes is the same in the fast-growing R.japonicum and in slow-growing cowpea Rhizobium. In NGR234 only one locus has detectable homology with known Hac and Nod genes of R.trifolii and even this is a restricted degree of homology. The other locus required for the expression of the nodulation

phenotype has not yet been identified, but this seems within reach with R-prime plasmids recently isolated from the Fix^- mutant U7 (M. Nayudu, personal communication). These plasmids must contain all the Sym plasmid-encoded nodulation genes, since they are able to confer a Nod^+ phenotype to the cured strain, ANU265, which lacks the Sym plasmid. Further DNA cloning and transposon mutagenesis using these plasmids should lead to the identification of other genes on the Sym plasmid which are required for nodulation. It may even be possible to isolate real host-range mutants, which are proficient in nodulation with one group of plants but are Nod^- on another type of plant.

The work in this thesis has led to the isolation of a mobilisable Sym plasmid, Tn5 induced mutants and cloned regions essential for the nodulation of cowpea plants, Leucaena leucocephala, and Parasponia. With these materials it will be possible to investigate the requirements for the expression of the broad-host-range symbiotic capacity of strain NGR234 from the level of biology to the DNA sequence.

APPENDIX ONE

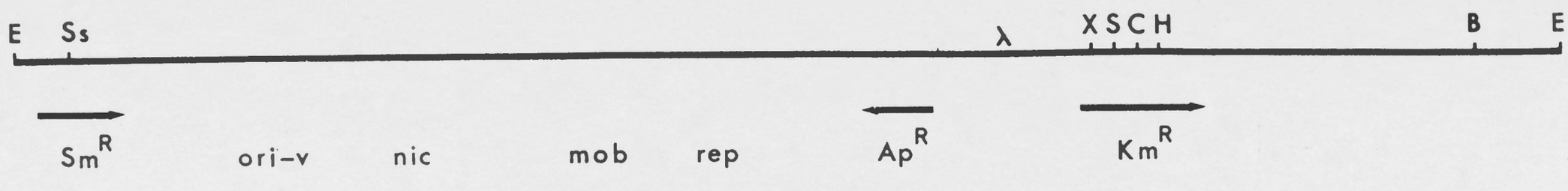
pNAM2, A VECTOR PLASMID CONSTRUCTED DURING THIS THESIS

A vector plasmid was constucted to provide a means of easily cloning restriction fragments on a vector which could replicate in Rhizobium. Several plasmids derived from the Pseudomonas plasmid, RSF1010, have been described by Bagdasarian et al. (1979). pNAM2 was made by rearranging two of these plasmids. In short, a small BstEII fragment, which contains an Ap^R determinant and a bacteriophage lambda cos site, was cloned from the plasmid pKT247 into the unique BstEII site in pKT230. The resultant plasmid, pNAM2, has Ap^R, Km^R and a low level Sm^R as markers. The plasmid is 14kb long and has unique sites for several restriction enzymes, which include XhoI, HindIII, ClaI, XmaI, BamHI, SstI and EcoRI. The XhoI, HindIII, XmaI and ClaI sites are in the Km^R gene (Fig. 4.1). The plasmid theoretically can be used for cosmid cloning in DNA in bacteriophage lambda heads as described by Hohn and Murray (1977). The plasmid was checked for maintenance in Rhizobium by mobilising into ANU265 and assaying for the plasmid through non-selective sub-culture. No loss of pNAM2 could be detected among 100 colonies assayed for Km^R after five serial sub-cultures. Athough the plasmid could be mobilised by the helper

plasmid pRK2013 at a high frequency (about 10^{-1}) it was also capable of self-transfer at about 10^{-4}.

Figure A.1. Map of the plasmid pNAM2 showing unique restriction endonuclease sites and relevant genes. Letters means: E, EcoRI; Ss, SstI; X, XhoI; S, SmaI (XmaI); C, ClaI; H, HindIII; B, BamHI. Genes are: ori-V is the origin of replication; nic is a specific nuclease site associated with mobilisability; mob is the origin of transfer; rep is a region essential for replication. The antibitic resitance genes are indicated.

pNAM2



LITERATURE CITED

Akkermans, A.D.L., S. Abdulkadir and M.J. Trinick. (1978). N_2-Fixing root nodules in Ulmaceae: Parasponia or (and) Trema spp.? Plant and Soil 49: 711-715.

Allen, E.K. and O.N. Allen. (1981). The Leguminosae: A source book of characteristics, uses and nodulation. (Macmillan Publishing Ltd., London).

Appelbaum, E.K. and N.A. Chartrain. (1983). Analysis of plasmids in fast-growing rhizobia that nodulate soybeans. In abstracts of"9th Nth. American Rhizobium Meeting." (ed) A.P.J. Eaglesham, T.A. LaRue and A.A. Szalay. (Cornell University Press). p 24.

Appelbaum, E.R., R. Johansen and N.A. Chartrain. (1984). Identification of plasmids carrying symbiotic genes in fast-growing R.japonicum using DNA hybridisation and Tn5 mutagenesis. In "Advances in Nitrogen Fixation." (ed) C. Veeger and W.E. Newton. (Martinus Nijhoff/Dr W. Junk publishers, The Hague, Boston, Lancaster and Pudoc, Wageningen). p 670.

Appelby, C.A., J.D. Tjepkema and M.J. Trinick. (1983). Hemoglobin in a nonleguminous plant, Parasponia: Possible genetic origin and function in nitrogen fixation. Science. 220: 951-953.

★Appelby, C.A., Y.P. Chen, W.F. Dudman, T.J. Higgins, A.S. Inglis, A.A. Kortt, W.D. Sutton and M.J. Trinick. (1984). Parasponia hemoglobin: Implications for the origin of plant hemoglobin. In "Advances in Nitrogen Fixation." (ed) C. Veeger and W.E. Newton. (Martinus Nijhoff/Dr W. Junk, The Hague, Boston, Lancaster and Pudoc, Wageningen). p 493.

Arthur, A. and D. Sherrat. (1979). Dissection of the transposition process: a transposon-encoded site -specific recombination system. Mol. Gen. Genet. 175: 267-274.

★Badenoch-Jones, J., M.A. Djordjevic, P. Gresshoff, B. Rolfe, J. Shine and W. Zurkowski. (1981). Molecular properties of plasmids in Rhizobium strains. In "Current Perspectives in Nitrogen Fixation." (ed) A.H. Gibson, W.E. Newton. (Australian Academy Science, Canberra). p 410.

Badenoch-Jones, J., R.E. Summons, M.A. Djordjevic, J. Shine, D.S. Letham and B.G. Rolfe. (1982). Mass spectrometric quantification of indole-3-acetic acid in Rhizobium culture supernatants: relation to root hair curling and nodule initiation. Appl. Environ. Microbiol. 44: 275-280.

Badenoch-Jones, J., C. Bates, K. Scott, B. Rolfe and J. Shine. (1984). Nitrogenase genes in the fast-growing broad-host range Rhizobium strain ANU240. In "Advances in Nitrogen Fixation." (ed) C. Veeger and W.E. Newton. (Martinus Nijhoff/Dr W. Junk Publishers, The Hague, Boston, Lancaster and Pudoc, Wageningen). p 671.

Bagdasarian, M., M.M. Bagdasarian, S. Coleman and K.N. Timmis. (1979). New vector plasmids for cloning in Pseudomonas. In "Plasmids of medical, environmental and commercial importance." (ed) K.N. Timmis and A. Puhler. (Elsevier/North Holland Biomedical Press, Amsterdam, New York). pp 411-422.

Banfalvi, Z., V. Sakanyan, C. Koncz, A. Kiss, I. Dusha and A. Kondorosi. (1981). Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid in R.meliloti. Mol. Gen. Genet. 184: 318-325.

Bauer, W.D. (1981). The infection of legumes by rhizobia. Ann. Rev. Pl. Physiol. 32: 407-449.

Becking, J.H. (1977). Dinitrogen fixing associations in higher plants other than legumes. In "A treatise in Dinitrogen Fixation Section III." (ed) R.W.F. Silver and W.S. Silver. (John Wiley and Son, New York). pp 185-275.

★Benton, W.D. and R.W. Davis. (1977). Screening lambda gt recombinant clones by hybridisation to single plaques in situ. Science. 196: 180-182.

Berg, D.E. (1977). Insertion and excision of the transposable resistance determinant Tn5. In "DNA insertion elements, plasmids and episomes." (ed) A.I. Bakhar, J.A. Shapiro and S.L. Adhya. (Cold Spring Harbor Press, Cold Spring Harbor, New York). pp 205-212.

Berg, D.E., A. Weiss and L. Crossland. (1980). Polarity of Tn5-insertion mutations in Escherichia coli. J. Bacteriol. 142: 439-446.

Bergersen, F.J. (1961). The growth of Rhizobium in synthetic media. Aust. J. Biol. Sci. 14: 349-360.

★Bergersen, F.J. (1973). Symbiotic nitrogen fixation by legumes. In "Chemistry and Biochemistry of Herbage". Vol 2, (ed) G.W. Butler, R.W. Bailey. (Academic Press, London). pp 189-226.

Beringer, J.E. (1974). R Factor transfer in Rhizobium leguminosarum. J. Gen. Microbiol. 84: 188-189.

★Beringer, J.E. and D.A. Hopwood. (1976). Chromosomal recombination and mapping in Rhizobium leguminosarum. Nature. 264: 291-293.

★Beringer, J.E., A.W.B. Johnston and B. Wells. (1977). The isolation of conditional ineffective mutants of Rhizobium. J. Gen. Microbiol. 98: 339-343.

Beringer, J.E., J.L. Beynon, A.V. Buchanan-Wollaston and A.W.B. Johnston. (1978a). Transfer of the drug-resistance transposon Tn5 to Rhizobium. Nature. 276: 633-634.

Beringer, J.E., S.A. Hoggan and A.W.B. Johnston. (1978b). Linkage mapping in Rhizobium leguminosarum by means of R plasmid-mediated recombination. J. Gen. Microbiol. 104: 201-207.

★Beringer, J.E., N.J. Brewin, A.W.B. Johnston, H.M. Schulman and D.A. Hopwood. (1979). The Rhizobium-legume symbiosis. Proc. R. Soc. London. 204: 219-233.

★Beringer, J.E., A.W.B. Johnston, and A. Kondorosi. (1982). Genetic maps of Rhizobium leguminosarum, R.meliloti, R.phaseoli, and R.trifolii. In "Genetic Maps" Vol. 2 (ed) S.J. O'Brien. Cold Spring Harbor Laboratory. pp 130-132.

Beynon, J.L., J.E. Beringer and A.W.B. Johnston. (1980). Plasmids and host-range in Rhizobium leguminosarum and Rhizobium phaseoli. J. Gen. Microbiol. 120: 421-429.

Bhuvaneswari, T.V., B.G. Turgeon and W.D. Bauer. (1980). Early events in the infection of soybean (Glycine max L. Merr) by Rhizobium japonicum I. Localization of infectible root cells. Plant Physiol. 66: 1027-1031.

Bhuvaneswari, T.V., A.A. Bhagwat and W.D. Bauer. (1981). Transient susceptibility of root cells in 4 common legumes to nodulation by rhizobia. Plant Physiol. 68: 1144-1149.

Birnboim, H.C. and J. Doly. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

Bolivar, F., R.L. Rodriguez, P.J. Greene, M.C. Betlach, H.L. Heyneker, H.W. Boyer, J.H. Crosa and S. Falkow. (1977). Construction and characterization of new cloning vehicles II. A multipurpose cloning system. Gene. 2: 95-113.

★Bolivar, F. (1978). Construction and characterization of new cloning vehicles III. Derivatives of plasmid pBR322 carrying unique EcoRI generated recombinant DNA molecules. Gene. 4: 121-136.

Brewin, N.J., J.E. Beringer, A.V. Buchanan-Wollaston, A.W.B. Johnston and P.R. Hirsch. (1980a). Transfer of symbiotic genes with bacteriocinogenic plasmids in Rhizobium leguminosarum. J. Gen. Microbiol. 116: 261-270.

Brewin, N.J., J.E. Beringer and A.W.B. Johnston. (1980b). Plasmid-mediated transfer of host-range specificity. J. Gen. Microbiol. 120: 413-420.

★Buchanan-Wollaston, A.V., J.E. Beringer, N.J. Brewin, P.R. Hirsch and A.W.B. Johnston. (1980). Isolation of symbiotically defective mutants in Rhizobium leguminosarum by insertion of the transposon Tn5 into a transmissible plasmid. Mol. Gen. Genet. 178: 185-190.

★Bukhari, A.I. and L. Ambrosio. (1978). The invertible segment of bacteriophage Mu DNA determines the absorption properties of Mu particles. Nature. 271: 575-578.

★Burkhardt, H.J., G. Reiss, and A. Puhler. (1979). Relationship of group P1 plasmids revealed by heteroduplex experiments: RP1,RP4,R68 and RK2 are identical. J. Gen. Microbiol. 114:341-348.

★Calos, M.P. and J.H. Miller. (1980). Transposable elements. Cell. 20: 579-595.

Callaham, D.A. and J.G. Torrey. (1981). A structural basis for infection of root hairs of Trifolium repens by Rhizobium. Can. J. Botany. 59: 1647-1664.

★Casadesus, J. and J. Olivares. (1979). Rough and fine linkage mapping of the Rhizobium meliloti chromosome. Mol. Gen. Genet. 174: 203-209.

Casadesus, J., E. Ianez and J. Olivares. (1980). Transposition of Tn1 to the Rhizobium meliloti genome. Mol. Gen. Genet. 180: 405-410.

Casse, F., C. Boucher, J.S. Julliot, M. Michel and J. Denarie. (1979). Identification and characterization of large plasmids in Rhizobium meliloti using agarose gel electrophoresis. J. Gen. Microbiol. 113: 229-242.

Cen, Y.H., G.L. Bender, M.J. Trinick, N.A. Morrison, K.F. Scott, P.M. Gresshoff, J. Shine and B.G. Rolfe. (1982). Transposon mutagenesis in rhizobia which can nodulate both legumes and the non-legume Parasponia. Appl. Environ. Microbiol. 43: 233-236.

Chakravorty, A.K., W. Zurkowski, J. Shine and B.G. Rolfe. (1982). Symbiotic nitrogen fixation: Molecular cloning of Rhizobium genes involved in exopolysaccharide synthesis and effective nodulation. J. Mol. Appl. Genet. 1: 585-596.

Chandler, M.R. (1978). Some observations of Arachis hypogeae L. nodulation by Rhizobium. J. Exp. Bot. 29: 749-755.

Chandler, M.R. R.A. Date and R.J. Roughley. (1982). Infection and root nodule development in Stylosanthes species by Rhizobium. J. Exp. Bot. 33: 47-57.

Chang, A.C.Y. and S.N. Cohen. (1978). Construction and characterisation of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134: 1141-1156.

★Chen, A.P.T. and D.A. Phillips. (1976). Attachment of Rhizobium to legume roots as the basis for specific interactions. Physiol. Plant. 38: 83-88.

★Chilton, M.D., M.H. Drummond, D.J. Merlo, D. Sciaky, A.L. Montoya, M.P. Gordon and E.W. Nester. (1977). Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell. 11: 263-271.

★Clewell, D.B. (1972). Nature of ColE1 plasmid replication in Esherichia coli in the presence of chloramphenicol. J. Bacteriol. 110: 667-676.

Corbin, D., L. Barran and G. Ditta. (1983). Organisation and expression of R.meliloti nitrogen fixation genes. Proc. Nat. Acad. Sci. 80: 3005-3009.

Coventry, D.R., M.J. Trinick and C.A. Appelby. (1976). A search for leghaemoglobin-compound in root nodules of Trema cannabina Lour. Biochem. Biophys. Acta. 420: 105-111.

★Currier, T.C. and E.W. Nester. (1976). Evidence for diverse types of large plasmids in tumor inducing strains of Agrobacterium. J. Bacteriol. 126: 157-165.

Dart, P.J. (1977). Infection and development of leguminous nodules. In "Treatise on Dinitrogen Fixation. Section III. Biology." (ed) R.W.F. Hardy and W.S. Silver. (John Wiley and Sons, New York). pp 367-472.

Datta, N., R.W. Hedges, E.J. Shaw, R.B. Sykes and M.H. Richmond. (1971). Properties of an R factor from Pseudomonas aeruginosa. J. Bacteriol. 108: 1244-1249.

Dazzo, F.B. and D.H. Hubbell. (1975a). Concanavalin A: antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association. Plant and Soil. 43: 713-717.

Dazzo, F.B. and D.H. Hubbell. (1975b). Cross reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association. Appl. Microbiol. 30: 1017-1033.

★Dazzo, F.B., M.R. Urbano and W.J. Brill. (1979). Transient appearance of lectin receptors on Rhizobium trifolii. Curr. Microbiol. 2: 15-20.

Dazzo, F.B. and G.L. Truchet. (1983). Interactions of lectins and their saccharide receptors in the Rhizobium-legume symbiosis. J. Membrane Biol. 73: 1-16.

Denarie, J., G. Truchet and B. Bergeron. (1976). Effects of some mutations of symbiotic properties of Rhizobium. In "Symbiotic Nitrogen Fixation in Plants." (ed) P.S. Nutman. (Cambridge University Press, Cambridge). pp 47-61.

★Denarie, J., C. Rosenberg, P. Boistard, G. Truchet and F. Casse-Delbart. (1981). Plasmid control of symbiotic properties in Rhizobium meliloti. In "Current Perspectives in Nitrogen Fixation." (ed) A.H. Gibson and W.E. Newton (Australian Acadamy Science, Canberra). pp 137-141.

★Dilworth, M.J. and C.A. Appleby. (1979). Leghaemoglobin and Rhizobium haemoproteins. In "A Treatise on Dinitrogen Fixation." (ed) R.W.F. Hardy, F. Bottomley and R.C. Burns. (John Wiley and Sons, New York). pp 691-764.

Ditta, G., S. Stanfield, D. Corbin and D.R. Helinski. (1981). Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. Proc. Nat. Acad. Sci. 77: 560-568.

Dixon, R.A. and J.R. Postgate. (1971). Transfer of nitrogen fixation genes by conjugation in Klebsiella pneumoniae. Nature. 234: 47-48.

Dixon, R., F. Cannon and A. Kondorosi. (1976). Construction of a P plasmid carrying nitrogen fixation genes from Klebsiella pneumoniae. Nature. 260: 268-271.

★Djordjevic, M.A., W. Zurkowski and B.G. Rolfe. (1982). Plasmids and stability of symbiotic properties of Rhizobium trifolii. J. Bacteriol. 151: 560-568.

Djordjevic, M.A., W. Zurkowski, J. Shine and B.G. Rolfe. (1983). Sym plasmid transfer to symbiotic mutants of Rhizobium trifolii, Rhizobium leguminosarum and Rhizobium meliloti. J. Bacteriol. 156: 1035-1045.

Downie, J.A., G. Hombrecher, Q.-S. Ma, C.D. Knight and B. Wells. (1983a). Cloned nodulation genes of Rhizobium leguminosarum determine host-range specificity. Mol. Gen. Genet. 190: 359-365.

Downie, J.A., Q.-S. Ma, C.D. Knight, G. Hombrecher and A.W.B. Johnston. (1983b). Cloning of the symbiotic region of Rhizobium leguminosarum: the nodulation genes are between the nitrogenase genes and a nifA-like gene. EMBO Journal. 2: 947-952.

★Dreyfus, B.L. and Y.R. Dommergues. (1981). Stem nodules on the tropical legume Sesbania rostrata. In "Current Perspectives in Nitrogen Fixation." (ed) A.H. Gibson and W.E. Newton. (Australian Academy Science, Canberra). p 471.

★Drummond, H.M., M.P. Gordon, E.W. Nester and M.D. Chilton. (1977). Foreign DNA of bacterial plasmid origin is transcribed in crown gall tumours. Nature. 269: 535-536.

Duncan, M.J. (1981). Properties of Tn5-induced carbohydrate mutants in Rhizobium meliloti. J. Gen. Microbiol. 122: 61-67

Eckhardt, T. (1978). A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid. 1: 584-588.

Fahraeus, G. (1957). The infection of clover root hairs by nodule bacteria, studied by a simple slide technique. J. Gen. Microbiol. 16: 374-381.

Figurski, D.H. and D.R. Helinski. (1979). Replication of an origin containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. 76: 1648-1652.

Forrai, T., E. Vincze, Z. Banfalvi, G.B. Kiss, G.S. Randhawa and A. Kondorosi. (1983). Localisation of symbiotic mutants in Rhizobium meliloti. J. Bacteriol. 153: 635-643.

Fuhrmann, M., K. Kaluza and H. Hennecke. (1984). In Rhizobium japonicum the nifH and nifDK genes are separated. In "Advances in Nitrogen Fixation." (ed) C. Veeger and W.E. Newton. (Martinus Nijhoff/Dr W. Junk Publishers, The Hague, Boston, Lancaster and Pudoc, Wageningen). p 682.

Fuller, F., P.W. Kunster, T. Nguyen and D.P.S. Verma. (1983). Soybean nodulin genes: Analysis of cDNA clones reveals several major tissue-specific sequences in nitrogen-fixing root nodules. Proc. Nat. Acad. Sci. 80: 2594-2598.

Gamborg, O.L. and D. Eveleigh. (1968). Detection of gluconases in suspension cultures of barley. Can. J. Biochem. 46: 417-422.

Gibson, A.H. (1963). Physical environment and symbiotic nitrogen fixation. I The effect of root temperature on recently nodulated Trifolium subterraneum L. plants. Aust. J. Biol. Sci. 16: 28-42.

Goodchild, D.J. (1977). The ultrastructure of root nodules in relation to nitrogen fixation. Int. Rev. Cytol., Suppl. 6: 235-288.

Goldblatt, P. 1981. Cytology and the phylogeny of Leguminosae. In "Advances in Legume Systematics, Part I" (ed) R. M. Pohill and P. H. Raven (Royal Botanic Gardens, Kew, England). pp 427-432.

Graham, P.H. (1964). The application of computer techniques to the taxonomy of the root-nodule bacteria of legumes. J. Gen. Microbiol. 35: 511-517.

Gresshoff, P.M., M.L. Skotnicki, J.F. Eadie and B.G. Rolfe. (1977). Viability of Rhizobium trifolii bacteroids from clover root nodules. Plant Sci. Lett. 10: 299-304.

Gresshoff, P.M. and B.G. Rolfe. (1978). Viability of Rhizobium bacteroids isolated from soybean nodule protoplasts. Planta. 142: 329-333.

Grunstein, M. and J. Wallis. (1979). Colony hybridisation. In "Methods in Enzymology." Vol.68 (ed) R. Wu. (Academic Press, New York). pp 379-389.

Haas, D. and Holloway, B.W. (1976). R Factor variants with enhanced sex factor activity in Pseudomonas aeruginosa. Mol. Gen. Genet. 144: 243-251.

Hanks, J.F., K. Schubert and N.E. Tolbert. (1983). Isolation and characterization of infected and uninfected cells from soybean nodules - role of uninfected cells in ureide synthesis. Plant Physiol. 71: 869-873.

Hennecke, H. (1981). Recombinant plasmids carrying nitrogen fixation genes from Rhizobium japonicum. Nature. 291: 354-355.

★Henson, I.E. and C.T. Wheeler. (1976). Hormones in plants bearing nitrogen fixing root nodules: The distribution of cytokinins in Vicia faba L. New Phytol. 76: 433-439.

Higashi, S. (1967). Transfer of white clover infectivity of Rhizobium trifolii to Rhizobium phaseoli as mediated by an episomic factor. J. Gen. Appl. Microbiol. 13: 391-403.

★Hirsch, P.R. (1979). Plasmid-determined bacteriocin production by Rhizobium leguminosarum. J. Gen. Microbiol. 113: 219-228.

Hirsch, P.R., M. van Montagu, A.W.B. Johnston, N.J. Brewin and J. Schell. (1980). Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of Rhizobium leguminosarum. J. Gen. Microbiol. 120: 403-412.

Hirsch, A.M., S.R. Long, M. Bang, N. Haskins and F.M. Ausubel. (1982). Structural studies of alfalfa roots infected with nodulation mutants of Rhizobium meliloti. J. Bacteriol. 151: 411-419.

Hirschel, B.J., D.J. Galas and M. Chandler. (1982). Cointegrate formation by Tn5, but not transposition, is dependent on recA. Proc. Nat. Acad. Sci. 79: 4530-4534.

Hohn, B. and K. Murray. (1977). Packaging recombinant DNA molecules into bacteriophage particles in vitro. Proc. Nat. Acad. Sci. 74: 3259.

Holliday, R. (1956). A new method for the identification of biochemical mutants of micro-organisms. Nature. 178: 987.

Holloway, B.W. (1979). Plasmids that mobilise bacterial chromosomes. Plasmid. 2: 1-19.

Holmes, D.S. and M. Quigley. (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114: 193-197.

Hombrecher, G., N.J. Brewin and A.W.B. Johnston. (1981). Linkage of genes for nitrogenase and nodulation ability on plasmids in Rhizobium leguminosarum and R.phaseoli. Mol. Gen. Genet. 182: 133-136.

Hooykaas, P.J.J., A.A.N. van Brussel, H. den Dulk-Ras, G.M.S. van Slogteren and R.A. Schilperoort. (1981). Sym plasmid of Rhizobium trifolii expressed in different rhizobial species and Agrobacterium tumefaciens. Nature. 291: 351-353.

★Hooykaas, P.J.J., F.G.M. Snijdewint and R.A. Schilperoort. (1982). Identification of the Sym plasmid of Rhizobium leguminosarum strain 1001 and its transfer to and expression in other rhizobia and Agrobacterium tumefaciens. Plasmid. 8: 73-82.

Hughes, J.E. (1981) Molecular cloning and characterisation of symbiotic genes from Rhizobium trifolii. PhD. Thesis, Australian National University, Canberra.

Jarvis, B.D.W., K.F. Scott, J.E. Hughes, M. Djordjevic, B.G. Rolfe and J. Shine. (1983). Conservation of genetic information between different Rhizobium species. Can. J. Microbiol. 29: 200-209.

Johnston, A.W.B. and J.E. Beringer. (1977). Chromosomal recombination between Rhizobium species. Nature. 267: 611-613.

Johnston, A.W.B., J.L. Beynon, A.V. Buchanan-Wollaston, S.M. Setchell, P.R. Hirsch and J.E. Beringer. (1978). High frequency transfer of nodulating ability between strains and species of Rhizobium. Nature. 276: 634-636.

Jordan, D.C. (1982). Transfer of Rhizobium japonicum to Bradyrhizobium, a new genus a slow-growing, root nodule bacterium from leguminous plants. Int. J. Syst. Bacteriol. 32: 136-139.

Jorgensen, R.A., S.J. Rothstein and W.S. Reznikoff. (1979). A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol. Gen. Genet. 177: 65-72.

Kado, C.I. and S.T. Liu. (1981) Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145: 1365-1373.

★Kehoe, M., R. Sellwood, P.Shipley and G. Dougan. (1981). Genetic analysis of K88-mediated adhesion of enterotoxigenic Escherichia coli. Nature. 291: 122-126.

Keyser, H.H., B.B. Bohlool, T.S. Hu and D.F. Weber. (1981) Fast-growing rhizobia isolated from root nodules of soybean. Proc. Nat. Acad. Sci. 215: 1631-1632.

Kleckner, N. (1977). Translocatable elements in prokaryotes. Cell. 11: 11-23.

Kleckner, N. (1981). Transposable elements in prokaryotes. Ann. Rev. Genet. 15: 341-404.

★Kondorosi, A., G.B. Kiss, T. Forrai, E. Vincze and Z. Banfalvi. (1977). Circular linkage map of Rhizobium meliloti chromosome. Nature. 268: 525-527.

Kondorosi, A., E. Vincze, A.W.B. Johnston and J.E. Beringer. (1980). A comparison of three Rhizobium linkage maps. Mol. Gen. Genet. 178: 403-408.

Kondorosi, A., E. Kondorosi, C.E. Pankhurst, W.J. Broughton and Z. Banfalvi. (1982). Mobilisation of a Rhizobium meliloti megaplasmid carrying nodulation and nitrogen fixation genes into other rhizobia and Agrobacterium. Mol. Gen. Genet. 188: 433-439.

Kondorosi, E., Z. Banfalvi, C. Slaska-Kiss and A. Kondorosi. (1983). Analysis of symbiotic nitrogen fixation genes carried by Rhizobium meliloti megaplasmids. In "Plant Molecular Biology", UCLA Symposia on Molecular and Cellular Biology, New Series VolXII (ed) R.B. Goldberg. (A.R. Liss Inc., New York). pp 259-275.

Law, I.J., Y. Yamamoto, A.J. Mort and W.D. Bauer. (1982). Nodulation of soybean by Rhizobium japonicum mutants with altered capsule synthesis. Planta. 154: 100-109.

★Leong, S.A., G.S. Ditta and D.R. Helinski. (1982). Heme Biosynthesis in Rhizobium. J. Biol. Chem. 257: 8724-8730.

★Libbenga, K.R., F. van Iren, R.J. Bogers and M.F. Schraag-Lamers. (1973). The role of hormones and gradients in the initiation of cortex proliferation and nodule formation in Pisum sativum L. Planta. 114: 29-39.

★Lippincott, B.B. and J.A. Lippincott. (1969). Bacterial attachment of a specific wound site at an essential stage in tumour initiation by Agrobacterium tumefaciens. J. Bacteriol. 97: 620-628.

Liu, C.-P., P.W. Tucker, J.F. Mushinski and F.R. Blattner. (1980). Mapping of heavy gene chains for mouse immunoglobulins M and D. Science. 209: 1401-1403.

Long, S.R., W.J. Buikema and F.M. Ausubel. (1982). Cloning of Rhizobium meliloti nodulation genes by direct complementation of Nod⁻ mutants. Nature. 298: 485-488.

Ma, Q-S., A.W.B. Johnston, G. Hombrecher and J.A. Downie. (1982). Molecular genetics of mutants of Rhizobium leguminosarum which fail to fix nitrogen. Mol. Gen. Genet. 187: 166-171.

Marvel, D.J., G. Kuldau, A.M. Hirsch, J. Park, J.G. Torrey, and F.M. Ausubel. (1984). Cloning and characterization of a nodulation locus from Rhizobium Parasponium. In "Advances in Nitrogen Fixation Research" (ed) C. Veeger and W.E. Newton. (Martinus Nijhoff/ Dr W. Junk Publishers, The Hague, Boston, Lancaster and Pudoc, Wageningen). p 691.

★Meade, H.M. and E.R. Signer. (1977). Genetic mapping of Rhizobium meliloti. Proc. Nat. Acad. Sci. 74: 2076-2078.

Meade, H.M., S.R. Long, G.B. Ruvkun, S.E. Brown and F.M. Ausubel. (1982). Physical and genetic characterisation of symbiotic and auxotrophic mutants of Rhizobium meliloti induced by transposon Tn5 mutagenesis. J. Bacteriol. 149: 114-122.

Miller, J.H. (1972). Experiments in Molecular genetics. Cold Spring Harbor Symp Quant. Biol.

Morrison, D.A. (1979). Transformation and preservation of competent bacterial cells by freezing. In "Methods in Enzymology" Vol. 68 (ed) R. Wu (Academic Press, New York) pp 326-331.

Morrison, N.A., C.Y. Hau, M.J. Trinick, J. Shine and B.G. Rolfe. (1983). Heat curing of a Sym plasmid in a fast-growing Rhizobium species that is able to nodulate legumes and the non-legume Parasponia sp. J. Bacteriol. 153: 527-531.

Norris, D.O. (1956) Legumes in the Rhizobium symbiosis. Empire J. Exp. Agric. 24: 246-270.

★Nuti, M.P., A.M. Ledeboer, A.A. Lepidi and R.A. Schilperoort. (1977). Large plasmids in different Rhizobium species. J. Gen. Microbiol. 100: 241-248.

★Nuti, M.P., A.A. Lepidi, R.K. Prakash, R.A. Schilperoort and F.C. Cannon. (1979). Evidence for nitrogen fixation (nif) genes on indigenous Rhizobium plasmids. Nature. 282: 533-535.

Nutman, P.S. (1956). Genetics of symbiosis and nitrogen fixation in legumes. Proc. Roy. Soc. 172: 417-437.

O'Farrell, P.H., E. Kutter and M. Nakanishi. (1980). A restriction map of the bacteriophage T4 genome. Mol. Gen. Genet. 179: 421-435.

O'Gara, F., C. Casey and E. Bolton. (1982). Introduction and behaviour of transposons in Rhizobium spp. In "Proceedings 8th Nth American Rhizobium Conference" (ed) K.W. Clark and J.H.G. Stephens. (University of Manitoba Press, Canada). pp 13-14.

Ow, D.W. and F.M. Ausubel. (1983). Regulation of nitrogen metabolism genes by nifA gene product in Klebsiella pneumoniae. Nature. 301: 307-313.

Pain, A.N. (1979). Symbiotic properties of antibiotic -resistant and auxotrophic mutants of Rhizobium leguminosarum. J. Appl. Bacteriol. 47: 53-64.

Pankhurst, C.E. and E.A. Schwinghamer. (1974). Adenine requirement for nodulation of pea by an auxotrophic mutant of Rhizobium leguminosarum. Arch. Microbiol. 100: 177-184.

Pankhurst, C.E., D.B. Scott and C.W. Ronson. (1982). Correlation between rifampicin resistance of slow-growing Rhizobium strains and their ability to express nitrogenase in culture. FEMS Letts. 15: 137-139.

Pankhurst, C.E., W.J. Broughton, C. Bachem, E. Kondorosi and A. Kondorosi. (1983). Identification of Nitrogen fixation and Nodulation genes on a large plasmid from a broad-host-range Rhizobium spp. In "Molecular Genetics of the Bacteria Plant Interaction." (ed) A. Puhler (Springer-Verlag, Berlin, Heidelberg). pp 169-176.

Phillips, D.A. and J.G. Torrey. (1970). Cytokinin production by Rhizobium japonicum. Physiol. Plant. 23: 1057-1063.

Plazinski, J., P.J. Dart and B.G. Rolfe. (1983). Plasmid visualisation and nif gene location in nitrogen -fixing Azospirillum strains. J. Bacteriol. 155: 1429-1433.

Prakash, R.K., R.K. Schilperoort and M.P. Nuti. (1981). Large plasmids of fast-growing rhizobia : Homology studies and location of structural nitrogen fixation (nif) genes. J. Bacteriol. 145: 1129-1136.

Quinto, C., H. De La Vega, M. Flores, L. Fernandez, T. Ballado, G. Soberon and R. Palacios. (1982). Reiteration of nitrogen fixation gene sequences in Rhizobium phaseoli. Nature. 200: 724-726.

★Robertson, J.G., P. Lyttleton and C.E. Pankhurst. (1981). Preinfection and infection processes in the legume-*Rhizobium* symbiosis. In "Current Perspectives in Nitrogen Fixation" (ed) A.H. Gibson and W.E. Newton (Australian Academy Science, Canberra). pp 280-291.

★Robertson, J.G. and P. Lyttleton. (1982). Coated and smooth vesicles in the biogenesis of cell walls, plasma membranes, infection threads and peribacteroid membranes in root hairs and nodules of white clover. *J. Cell Sci.* 58: 63-78.

★Rolfe, B.G. and P.M. Gresshoff. (1980). *Rhizobium trifolii* mutant interactions during the establishment of nodulation in white clover. *Aust. J. Biol. Sci.* 33: 491-504.

Rolfe, B.G., P.M. Gresshoff and J. Shine. (1980). Rapid screening for symbiotic mutants of *Rhizobium* and white clover. *Plant Sci. Letters.* 19: 277-284.

Rolfe, B.G., M. Djordjevic, K.F. Scott, J.E. Hughes, J. Badenoch-Jones, P.M. Gresshoff, Y. Cen, W.F. Dudman, W. Zurkowski and J. Shine. (1981a). Analysis of nodule-forming ability of fast-growing Rhizobium strains. In "Current Perspectives in Nitrogen Fixation" (ed) A.H. Gibson and W.E. Newton (Australian Academy Science, Canberra). pp 142-145.

Rolfe, B.G., J. Shine, P.M. Gresshoff, K.F. Scott, M. Djordjevic, Y. Cen, J.E. Hughes, G.L. Bender, A. Chakravorty, W. Zurkowski, J.M. Watson, J. Badenoch-Jones, N.A. Morrison and M.J. Trinick. (1981b). Transposon induced mutants of fast and slow-growing rhizobia and the molecular cloning of symbiotic genes. In "Proceedings of the 8th Nth American Rhizobium Conference" (ed) K.W. Clark, J.H.G. Stephens (University of Manitoba Printing service). pp 34-54.

Rosenberg, C., P. Boistard, J. Denarie and F.L. Casse-Delbart. (1981). Genes controlling early and late functions in symbiosis are located on a megaplasmid in Rhizobium meliloti. Mol. Gen. Genet. 184: 326-333.

Ruvkun, G.B. and F.M. Ausubel. (1980). Interspecies homology of nitrogenase genes. Proc. Nat. Acad. Sci. 77: 191-195.

★Ruvkun, G.B. and F.M. Ausubel. (1981). Physical mapping of symbiotic nitrogen fixation genes in Rhizobium meliloti. In "Current Perspectives in Nitrogen Fixation" (ed) A.H. Gibson and W.E. Newton (Australian Academy Science, Canberra). pp 161-164.

Ruvkun, G.B., S.R. Long and H.M. Meade. (1982) Rhizobium meliloti insertion sequence that transposes preferentially into nitrogen fixation genes. J. Mol. Appl. Gen. 1: 405-418.

★Sanders, R.E., R.W. Carlson and P. Albersheim. (1978). A Rhizobium mutant incapable of nodulation and normal polysaccharide secretion. Nature. 271: 240-242.

★Sanders, R.E., E. Raleigh and E. Signer. (1981) Lack of correlation between extracellular polysaccharide and nodulation ability in Rhizobium. Nature. 292: 241-266.

Scherrer, A. and J. Denarie. (1971). Symbiotic properties of some auxotrophic mutants of Rhizobium meliloti and their prototrophic revertants. Plant and Soil. (Special Volume) 39-45.

Schofield, P.R., M.A. Djordjevic, B.G. Rolfe, J. Shine and J.M. Watson. (1983a). A molecular linkage map of nitrogenase and nodulation genes in Rhizobium trifolii. Mol. Gen. Genet. 192: 459-465.

Schofield, P.R., R.W. Ridge, M.A. Djordjevic, B.G. Rolfe, J. Shine and J.M. Watson. (1983b). Host-specific nodulation is encoded on a 14kb fragment in Rhizobium trifolii. Plant Mol. Biol. (in press).

Schwinghamer, E. A. 1977. Genetic aspects of nodulation and dinitrogen fixation by legumes: The microsymbiont. In "A treatise on dinitrogen fixation, Section III: Biology" (ed) W.S. Silver (John Wiley and Sons, New York). pp 577-622.

Scott, D.B. and C.W. Ronson. (1982). Identification and mobilisation by cointegrate formation of a nodulation plasmid in Rhizobium trifolii. J. Bacteriol. 151: 36-43.

Scott, K.F., J.E. Hughes, P.M. Gresshoff, J.E. Beringer, B.G. Rolfe and J. Shine. (1982). Molecular cloning of *Rhizobium trifolii* genes involved in symbiotic nitrogen fixation. *J. Mol. Appl. Gen.* 1: 315-326.

Scott, K.F., B.G. Rolfe and J. Shine. (1983a). Nitrogenase structural genes are unlinked in the nonlegume symbiont *Parasponia Rhizobium*. *DNA* 2: 141-148.

Scott, K.F., B.G. Rolfe and J. Shine. (1983b) Biological nitrogen fixation: Primary structure of the *Rhizobium trifolii* iron protein gene. *DNA* 2: 149-155.

Shapiro, J.A. (1979). Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. *Proc. Nat. Acad. Sci.* 76: 1933-1937.

Simon, R., G. Weber, W. Arnold and A. Puhler. (1982). Analysis of plasmid borne genes in *Rhizobium meliloti* by Tn*5* mutagenesis. In "Proceedings 8th Nth American *Rhizobium* Conference" (ed) K.W. Clarke and J.H.G. Stephens (University of Manitoba Press). pp 67-89.

Simon, R., U. Priefer, and A. Puhler. (1983). Vector plasmids for in-vivo and in-vitro manipulations of Gram-negative bacteria. In "Molecular Genetics of the Bacteria-Plant Interaction" (ed) A. Puhler. (Springer-Verlag, Berlin). pp 98-106.

Skotnicki, M.L. and B.G. Rolfe. (1979). Bacterial and bacteroid properties of mutants of Rhizobium trifolii strain T1 uncoupled in oxidative phosphorylation. Aust. J. Biol. Sci. 32: 501-517.

Soberon, K., L. Covarrubias and F. Bolivar. (1980). Construction and characterisation of new cloning vehicles IV Deletion derivatives of pBR322 and pBR325. Gene. 9: 287-305.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Stanisch, V.A. (1974). The properties and host range of male specific bacteriophage of Pseudomonas aeruginosa. J. Gen. Microbiol. 84: 332-335.

Starlinger, P. (1980). IS elements and transposons. Plasmid. 3: 241-259.

Sundaresan, V., D.W. Ow and F.M. Ausubel. (1983). Activation of Klebsiella pneumoniae and Rhizobium meliloti nitrogenase promoters by gln(ntr) regulatory proteins. Proc. Nat. Acad. Sci. 80: 4030-4034.

Trinick, M.J. (1968). Nodulation of tropical legumes. I Specificity in the Rhizobium of symbioses of Leucaena leucocephala. Expl. Agric. 4: 243-253.

Trinick, M.J. (1973). Symbiosis between Rhizobium and the non-legume Trema aspera. Nature. 244: 459-460.

Trinick, M.J. (1980). Relationships among the fast-growing rhizobia of Lablab purpureus, Leucaena leucocephala, Mimosa spp., Acacia farnesiana and Sesbania grandiflora and their affinities with other rhizobial groups. J. Appl. Bacteriol. 49: 39-53.

Trinick, M.J. and J. Galbraith. (1980). The Rhizobium requirements of the non-legume Parasponia in relationship to the cross-inoculation group concept of legumes. New Phytol. 86: 17-26.

Trinick, M.J., R.L. Rhodes and J.H. Galbraith. (1983). Competition between fast- and slow-growing tropical legume rhizobia for nodulation of Vigna unguiculata. Plant and Soil. 73: 105-115.

Turgeon, B.G. and W.D. Bauer. (1982). Early events in the infection of soybean by Rhizobium japonicum.Time course and cytology of the initial infection process. Can. J. Bot. 60: 152-161.

Van Brussel, A.A.N., T. Tak, A. Wetselaar, E. Pees, and C.A. Wijffelman. (1982). Small Leguminosae as test plants for nodulation of Rhizobium leguminosarum and other rhizobia and agrobacteria harbouring a leguminosarum Sym plasmid. Plant Sci. Lett. 27: 317-325.

Vance, C.P. (1983). Rhizobium infection and nodulation: a beneficial plant disease? Ann. Rev. Microbiol. 37: 399-424.

Van Larebeke, N., N. Engler, M. Holsters, S. Van den Elsecker, I. Zaenen, R.A.N. Schilperoort, and J. Schell. (1974). Large plasmid in Agrobacterium tumefaciens essential for crown gall-inducing activity. Nature. 252: 169-170.

Van Vliet, F., B. Silva, M. van Montagu and J. Schell. (1978). Transfer of RP4::Mu plasmids to Agrobacterium tumefaciens. Plasmid. 1: 446-455.

Vieira, J., and J. Messing. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene. 19: 259-268.

Vincent, J.M. (1970) A manual for the practical study of root-nodule bacteria. IBP handbook no. 15. (Blackwell Scientific Publications, Oxford).

★Vincent, J.M. (1974). Root-nodule symbiosis with Rhizobium. In: "The Biology of Nitrogen Fixation" (ed) A. Quispel. (North Holland Publishing Co., Amsterdam). pp 265-341.

Vincent, J.M. (1980). Factors controlling the legume-Rhizobium symbiosis. In "Nitrogen Fixation" Vol. 2 (ed) W.E. Newton and W.H. Orme-Johnson. (University Park Press, Baltimore). pp 103-129.

Wells, S.E. and L.D. Kuykendall. (1983). Tryptophan auxotrophs of Rhizobium japonicum. J. Bacteriol. 156: 1356-1358.

★Whithing, M.J. and M.J. Dilworth. (1974). Legume root nodule nitrogenase: Purification properties and studies on its genetic control. Biochim. et Biophys. Acta. 371: 337-351.

★Willets, N.S., C. Crowther and B.W. Holliday. (1981). The insertion sequence IS21 of R68.45 and the molecular basis for mobilisation of the bacterial chromosome. Plasmid. 6: 30-52.

Wilson, J.K. (1944). Over five hundred reasons for abandoning the cross-inoculation groups of the legumes. Soil Sci. 58: 61-69.

Wong, C.H., C.E. Pankhurst, A. Kondorosi and W. J. Broughton. (1983). Morphology of root nodules and nodule-like structures formed by Rhizobium and Agrobacterium strains containing a Rhizobium meliloti megaplasmid. J. Cell Biology. 97: 787-795.

Yao, P.Y. and J.M. Vincent. (1969). Host specificity in the root hair curling "factor" of Rhizobium spp. Aust. J. Biol. Sci. 22: 413-423.

★Yao, P.Y. and J.M. Vincent. (1976). Factors responsible for the curling and branching of clover root hairs by Rhizobium. Plant and Soil. 45: 1-16.★

Zimmerman, J.L., W.W. Szeto and F.M. Ausubel. (1983). Molecular characteristics of Tn5-induced symbiotic (Fix^-) mutants of Rhizobium meliloti. J. Bacteriol. 156: 1025-1034.

Zurkowski, W. (1980). Specific absorption of bacteria to clover root hairs, related to the presence of the plasmid pWZ2 in cells of Rhizobium trifolii. Microbios. 27: 27-32.

Zurkowski, W. (1981). Conjugational transfer of the nodulation-conferring plasmid pWZ2 in Rhizobium trifolii. Mol. Gen. Genet. 181: 522-524.

★Zurkowski, W. (1982). Molecular mechanism for the loss of nodulation properties of Rhizobium trifolii. J. Bacteriol. 150: 999-1007.★

Zurkowski, W. and Z. Lorkiewicz. (1978). Effective method for the isolation of non-nodulating mutants of Rhizobium trifolii. Genetic Research. 32: 311-314.

Zurkowski, W. and Z. Lorkiewicz. (1979). Plasmid mediated control of nodulation in Rhizobium trifolii. Archiv. Microbiol. 123: 195-201.

★ These references were consulted but were not cited in the text.